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Award Number: W81XWH-10-1-0774

TITLE: Theory-Driven Models for Correcting "Fight or Flight" Imbalance in Gulf War
Illness

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REPORT DATE: September 2013

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE September 2013		2. REPORT TYPE Final		3. DATES COVERED 1 September 2010 – 31 August 2013	
4. TITLE AND SUBTITLE Theory-Driven Models for Correcting "Fight or Flight" Imbalance in Gulf War Illness				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0774	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Gordon Broderick, Ph.D. E-Mail: gordon.broderick@ualberta.ca				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Alberta Edmonton, Alberta, Canada, T6G 2C8				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The objective of this study is to create a comprehensive engineering model of endocrine-immune interaction dynamics in order to identify (i) theoretical failure modes of the HPA-immune axis that align with GWI, and (ii) promising treatment strategies that exploit the regulatory dynamics of these systems to reset control of the HPA-immune axis to normal. We are currently transferring operations and this award to Nova Southeastern University, FL, to facilitate interactions with U.S. sponsors and collaborators. Dr. Craddock, now assistant professor at Nova, will continue in his role on this project. Work has focused on the refinement of the HPA-HPG-immune multi-axis model, its validation scheme and inclusion of Th17 and neurotransmission (NPY, acetylcholine, etc...) in the detailed immune model. Importantly we have developed an advanced prototype of a neuroinflammation model. Deployment to large-scale distributed computing platform has also advanced significantly.					
15. SUBJECT TERMS GWI; Hypothalamic-pituitary-adrenal (HPA) axis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	56	19b. TELEPHONE NUMBER (include area code)

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Introduction

The hypothalamic-pituitary-adrenal (HPA) axis controls the body's "fight or flight" response through a series of endocrine and immune signals directed at ensuring immediate survival and later re-establishing homeostasis. Changes in the tone of this response have been observed in veterans with Gulf War Illness (GWI). Studies report abnormal cell proliferation, impaired function and persistent oxidative stress in circulating immune cells of patients. Similarly dysregulation of the HPA axis includes hypersensitivity in cytokine feedback as well as suppression of cortisol and neurotransmitters responsible for mediating innate and adaptive immunity. This is further complicated by the impact on the HPA axis of a myriad of regulatory interactions both within and between i) the immune system and ii) the sex-hormone axis, the hypothalamic-pituitary-gonadal (HPG) axis.

We proposed that severe physical or psychological insult to the endocrine and immune systems can displace these from a normal regulatory equilibrium into a compromised stable state. This state is characterized by a self-perpetuating inflammatory response that involves regulatory imbalance between the HPA, HPG and immune axes. To explore the validity of this hypothesis our objective was to create comprehensive engineering models of endocrine-immune interaction dynamics in order to identify (i) theoretical failure modes of the endocrine-immune interplay that align with GWI, and (ii) promising treatment strategies that exploit the naturally occurring stable points of these systems.

Body.

At the time of our last update we had completed a re-assessment of the modeling approach and successfully identified, refined and deployed a discrete modeling paradigm enabling us to circumvent the significant gaps in the required parameter estimates exist in the literature. This new approach was described in greater detail in the previous report (September, 2012) (**Task 1**) and consists in an extension of the discrete logical network methodology proposed originally by Thomas et al. [1,2] and developed further by Mendoza and Xenarios [3]. *Importantly, this approach supports the seamless integration of kinetic information wherever available, be it simple sequential precedence, relative time scale or detailed dynamics.*

We have now completed the original scope of Task 1 through Task 5. However as a result of improvements in computational efficiency we have been able to extend the basic models beyond what was originally anticipated. With the concerted efforts of Dr. Craddock, new programming staff Mark Rice and Ryan del Rosario and research interns Simar Singh and Lundy McKibbin we have continued to: *(1) design and deploy a distributed version of the computing code, (2) increase the granularity of the multi-axis model and the implement a statistical scheme for model validation, (3) significantly extend the scope and increase fidelity of the detailed immune model, and (4) develop a prototype model of neuro-inflammation, and (5) designed and deployed a first prototype of the treatment optimization scheme.*

1. Continued algorithm development and speed-up. (Task 1). The core concept of the approach we have used is *connectivity*. Key biological regulatory processes have been translated into a set of discrete logic circuits. Analysis of these networks makes it possible to identify the number and type (e.g. oscillatory, etc...) of resting states as well as their molecular and cellular profile without detailed knowledge of response dynamics. Early implementations of this analysis were made in a high-level rapid-prototyping environment (Python) facilitating development but severely limiting computational performance. As mentioned previously, under this discrete formalism the number of model variables determines the total number of system-wide states such that a model of N state variables possesses 3^N states. As a result the number of total system-wide states increases rapidly as new state variable elements are added. Initially these calculations were encoded into a rapid-prototyping Python script that was used to search the above-mentioned network for stable equilibrium states (Version 0). Within a 24-hour threshold time (86,400 seconds) this version is capable of analyzing up to 14 variables (4,782,969 states) with a memory usage in the range of gigabytes (GB) (**Figure 1**). High-performance computing staff, programmers Rice and del Rosario, re-engineered the search algorithm and its implementation in several stages. First, the algorithm was directly re-coded in the C programming language (Version 1) as it is both memory-efficient and approximately 30 times faster than

Python. This increased performance enabled analysis of a 17-variable model (129,140,163 possible discrete states) in 24 hours at a memory usage in the megabyte (MB) range. Next, the serial algorithm was re-engineered through the introduction of ternary data structures to efficiently optimize memory usage and run time, and prepare the algorithm for parallel implementation (Version 2). Here, in the 24-hour threshold time, a 19-variable model (1,162,261,467 states) was analyzed at MB memory usage. Thirdly, the algorithm was implemented with parallel tasking (Version 3), using multiple levels of parallel threading (m0 to m4). Here we have successfully run a model with 23 variables (94,143,178,827 states) within a day using only MB's of memory. Finally, we have parallelized the code further with a supervisory layer based on message passing interface (MPI) (Version 4) to make full use of the high-performance computing resources on the University of Miami's Pegasus cluster. While performance measures are still being evaluated we have successfully analyzed a 25 variable circuit model (847,288,609,443 discrete states). Continued improvements to computational efficiency are ongoing.

2. Continued refinement of an integrated model of HPA-HPG-immune interaction (Task 3, Task 5).

We had previously extended our early model of HPA axis dynamics [4] by including feed-forward and feedback interactions with sex hormone regulation and immune response. A circuit model had been constructed that linked state variables across the HPA axis with hypothalamic-pituitary-gonadal (HPG) function in both men and women, as well as a coarse-grained mode of the immune system consisting of innate (IIR) and adaptive (AIR) immune components. A critical review of this model prompted us to: (i) re-assess the coarse-graining of the immune components (IIR and AIR aggregate nodes), increasing the level of detail to improve fidelity, and (ii) define and implement an alternate validation measure.

- *A modified multi-axis model.* In a first coarse iteration of the model, immune function was described simply in terms cytokine activity of the innate (IIR) and adaptive (AIR) immune responses. Here the aggregation of all adaptive immune response into the AIR node lacked the complexity needed to capture shifts between Th1 and Th2 activity. Further resolution was added to the immune model by separating the AIR into Th1 and Th2 activity and by adding both cell population activity, as well as cytokine signaling separately. In this modified immune module innate immune cells (ICells) produce cytokines that regulate the innate immune response (IIR) including interleukin (IL) -1, IL-6, IL-8, IL-12, IL-15, IL-23 and tumor necrosis factor alpha (TNF- α). These IIR signals serve to prime helper T cells towards a Th1 type adaptive immune response (T1Cell), producing Th1 pro-inflammatory cytokines (T1Cyt) including IL-2, interferon-gamma (IFN- γ), and tumor necrosis factor beta (TNF- β). This further activates ICells, while suppressing the Th2 adaptive immune response (T2Cell). The T2Cell node promotes the production of the Th2 anti-inflammatory cytokines (T2Cyt) IL-4, IL-5, IL-10 and IL-13, which serve to inhibit the activity of T1Cell and ICells. Interaction with the HPA axis is mediated by CORT suppressing ICell and Th1Cell activity, while IIR and Th1Cyt signals stimulate the HPA. Additionally, new interactions between the immune and HPG axis were included where Th1Cyt signals suppress GnRH and LH/FSH release and the dimorphic response of sex hormones TEST/ EST serve to induce Th1/Th2 activity.
- *A probabilistic measure of alignment with experimental data.* Alignment of model predictions with experimental data were previously assessed on the basis of discrete Hamming distance and visualized with a Sammon projection of the latter. In order to provide a more continuous measure of similarity or dissimilarity we have adopted a probabilistic measure proposed by Brown [5]. Here, we calculate the significance of alignment between experimental data and a given state predicted by the model using a meta-analysis technique that combines non-independent test statistics. Null probability p-values for individual variables are calculated using two-sample t-tests between ill subjects and healthy controls. To give the probability of obtaining the model value by chance 'right-handed' one-tailed tests are used when the model predicts a high state, 'left-handed' tests when predictions are low, and two-tailed tests when the prediction is a nominal value. These non-independent statistics are then combined into a chi-squared test statistic, which is scaled to $T = T_0/c$ with $2N/c$ degrees of freedom, where $c = \sigma^2/4N$. This statistic is then used in the scaled chi-squared distribution to determine the overall probability of obtaining the alignment by chance. The advantage of this method is that it accommodates for the dependence between variables, allows for a statement of confidence on alignment for each individual

model predicted state, and does not depend on the number of measureable markers allowing for direct comparison across models.

Details of this analysis and the final multi-axis model are described in Appendix A in manuscript recently submitted to PLoS One [6]. In brief, co-regulation of the HPA, HPG and immune systems has been described as a revised circuit model consisting of 14 state variables where each variable can assume one of three discrete states at any point in time: -1 (inhibited), 0 (nominal) and +1 (elevated). In this model the HPA axis continues to be described in terms of corticotropin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), cortisol (CORT) and cytosolic glucocorticoid receptors (GRs), which unlike membrane bound receptors, dimerize (GRD) (**Figure 2A-B**). HPG function is again described by the levels of gonadotropin-releasing hormone (GnRH), of luteinizing hormone (LH) as well as testosterone (TEST) in males (**Figure 2 C**) and estradiol (EST) in females (**Figure 2 D-G**). As before, the effects of gender merit special attention as testosterone (TEST) exhibits an inhibitory effect on the HPA axis while estrogen (EST) and progesterone can stimulate or suppress HPA activity depending on the phase of menstrual cycle. These components are integrated with the revised immune model described above.

Results of simulations conducted on the refined model can be summarized as follows:

- **Male subjects.** Inclusion of basic immune function and sex hormone regulation by the HPG axis with HPA function (HPA-GR-Immune-HPG model) *in male subjects* (**Figure 2C**) resulted in the emergence of 5 stable equilibrium states. Once again the first state was that of normal health (SS0). Low levels of ACTH and elevated expression of the glucocorticoid receptors GR and GRD characterized the second equilibrium state (SS1). The third stable state (SS2) exhibited suppressed innate and Th1 immune responses (low ICell, IIR, T1Cell, and T1Cyt), with increased Th2 activity (high T2Cell and T2Cyt). The fourth state (SS3) presented low ACTH, suppressed innate and Th1 immune activity (low ICell, IIR, T1Cell and T1Cyt), and elevated Th2 and glucocorticoid receptor activity (high GRD, GR, T2Cell and T2Cyt). The final state (SS4) displayed hypercortisolism, suppressed HPG activity and a shift towards the Th1 immune response (low T2Cell, T2Cyt, GnRH, LH/FSH and TEST/EST, and high CORT, GRD, GR, T1Cyt and T1Cell).
- **Female subjects.** In the specific case of positive feedback along the HPG axis and suppressive interaction with the HPA axis, the HPA-GR-Immune-HPG model for female subjects (**Figure 2F**) supported 11 steady states. In addition to 5 states equivalent to those obtained for the male subjects, we found new steady states that corresponded to suppressed HPA axis and innate immune response (low CRH, ACTH, CORT, ICell and IIR), while the HPG and anti-inflammatory response were elevated (high T2Cell, T2Cyt, GnRH, LH/FSH and EST). This combination occurred at each of the three low, nominal and high values for glucocorticoid receptor activity (GR/GRD) (SS5, SS6 and SS7, respectively). The final three additional states all supported suppressed HPA (CRH, ACTH, and CORT) and T1Cell activity, with elevated HPG activity (GnRH, LH/FSH and EST). These were again differentiated by their glucocorticoid receptor levels (GR/GRD at low (SS8), nominal (SS9) and high (SS10) values). Note that a stable steady state characterized by low cortisol levels was found *only for female subjects*.
- **Alignment with experimental data.** To validate these results the predicted steady states were first compared to steroid and cytokine levels recorded in male Gulf War veterans with GWI and healthy veterans (HCs) as part of a sister study [7]. As experimental measures for ACTH, GR, GRD, and immune cells populations were not available, certain steady states could not be distinguished and validated separately from one another. Comparison to the nominal states (SS0/SS1) showed poor alignment, with a null probability of $p=0.82$, suggesting that the GWI profile cannot be considered the same as normal behavior. The predicted states presenting a shift towards Th2 immune activation (SS2/SS3) showed improved alignment with a significance of $p=0.38$. However the final state (SS4) displaying hyper-cortisolism, low TEST and a shift towards Th1 immune activation yielded the best alignment with a null probability of $p=0.30$, supporting the notion of a more classical Th1 auto-immune signature with a concurrent (and perhaps stabilizing) endocrine component in GWI.

As a much greater proportion of women than men are affected by CFS, we compared the predicted steady states identified with the female model to experimental data collected under two compatible

studies [8,9]. Alignment with the baseline nominal setting in measureable variables (SS0/SS1) was poor, $p=0.83$, reinforcing that CFS is distinctly different from normal regulatory behavior. The Th2-shifted immune profiles predicted by the model (SS2/SS3) showed a significant alignment with the measured signature ($p=0.04$), suggesting that Th2 activation in CFS may at least in part be supported by homeostatic drive. This emphasized further by a low degree of alignment with the Th1 immune activated state, with hypercortisolism, and low EST ($p=0.28$). Improved alignment was found with states with a shift towards Th2, coupled with hypocortisolism, and high EST (SS5/SS6/SS7) ($p=0.02$). States presenting with only hypocortisolism and high EST, and no immune activation (SS8/SS9/SS10) aligned very weakly with the measured profile ($p=0.60$), suggesting that hypocortisolism, increased EST, and Th2 activation in combination are key CFS profile features that might owe at least part of their persistence to basic homeostatic control.

3. Refinement of detailed immune circuitry including Th17 and neurotransmission (Task 2, 3 and 5).

Based on the work of Folcik et al. (2007, 2011) [10,11] and an extensive review of recent literature, we constructed an initial wiring diagram describing cytokine signaling between immune cell populations [12] (see excerpts of manuscript in preparation; Appendix B in 2012 annual report). We have now extended this first detailed model of immune signaling at two levels of granularity:

- Addition of Treg and Th17 components to aggregate model of cytokine signaling. The specific cytokines supported in the initial model included interleukin (IL)-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-23, IL-27, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α . In order to improve computational efficiency, cytokines were grouped according to their dominant action into either a monokine (MK) or cytokine (CK) group (Folcik et al., 2011)[11]. We have now extended the model to include the actions of IL-17, 21 and 23 as well as TGF- β . The extended model also includes T regulatory (Treg), Th17 and activated Th17(23) cell populations as well as IgA and IgG antibody classes. This updated version of the cytokine signaling network includes as before the effects of stress and sex hormones for male subjects only at this time (**Figure 3**). Results of our stability analysis on this second-generation model that can be summarized as follows:
 - Stable immune response modes in male subjects. Application of this discrete dynamical analysis to the detailed endocrine-immune network yielded three predicted stable steady states. As always, the first state was that of normal health (SS0). The second stable state (SS1) presented with low anti-inflammatory cytokines (CK2), low testosterone, and suppressed NK cell activity, Th1 and Th2 immune cell activity, accompanied by elevated Th1 inflammatory cytokines (CK1), high cortisol levels, and increased cytotoxic T lymphocyte (CTL) and Treg cell activity. The third stable attractor (SS2) also displayed low NK cell activity and testosterone levels, with elevated cortisol, CK1 and CTL activity. However this state presented a different immune profile characterized by low TGF- β levels, elevated monocyte cytokines (MK1, MK2, MK6, MK21), and increased Th17 activity (CK17, Th17(23), TH17b). These results are consistent with the findings of the integrated HPA-HPG-immune model discussed above, but with added resolution in terms of immune function, which indicates alternate equilibria defined by different stable levels of regulatory T cell activity, or Th17 immune response.
 - Alignment with experimental data. Once again, these predicted steady states were compared with experimental data used in Section 1 [7-9]. We found alignment of GWI with the healthy reference state (SS0) corresponded to a null probability of $p=0.87$, indicating very poor alignment. Improved alignment was observed with SS1 ($p=0.30$). This is comparable to the results found for the high CORT, low TEST, elevated Th1 response state using the integrated HPA-HPG-Immune model described previously (section 2). Further improvement was found when comparing GWI to the final predicted stable state (SS2) ($p=0.12$), suggesting that chronic Th17 activation, hypercortisolism, and low TEST observed in this illness may persist in part as a result of homeostatic drive. For male CFS subjects we found comparably poor alignment with the reference baseline state SS0 ($p=0.76$). However, alignment with state SS1 ($p=0.16$) was dramatically different from GWI, emphasizing the distinct nature of CFS. Distinct as they may be, these illnesses nonetheless share some common components that our group has begun to

delineate at the level of specific pathways [13]. Consistent with this we found comparable alignment of CFS and GWI with SS2 ($p=0.12$) albeit for slightly different reasons. Thus, while GWI aligns best with Th17 dysregulation, CFS alignment suggests slightly different imbalance of Th17 and/or Treg response.

- *High fidelity model of individual cytokine actions.* Improved alignment with the clinical data can be accomplished by including additional key interactions in the model regulatory network, and by increasing resolution of the model in terms of the state variables represented. Key interactions located outside the immune network include critical neurotransmitters linking the brain and central nervous system with the HPA axis and the immune system. The neurotransmitters norepinephrine (NorEpi), and acetylcholine (ACh) are significant regulators of cytokine production, and therefore immune function. Neuropeptide Y (NPY) is also key component of the stress response, and its subsequent effects on the immune system. The latter has now been shown to play a significant role in CFS [14]. These messengers have now been included in a more refined model of the immune system and its interface with neurotransmission (**Figure 4**). Their effect on the immune system however is not simple. In the previous immune model several cytokines were aggregated into groups. NorEpi, ACh and NPY were found to have differing effects on the production of cytokines within individual groups. To accommodate these varied responses several of the aggregate nodes were separated into their individual constituent entities increasing the resolution, and complexity, of the extended immune model. The overall resulting high fidelity model of the extended immune system, including HPA, HPG and CNS inputs is shown in **Figure 4**. A first analysis has shown the following:
 - *Preliminary stable immune response modes in male subjects.* Discrete logical analysis of the preliminary high fidelity extended immune model produced two steady states. Normal health characterized the first state (SS0), while the second state (SS1) presented with low IL-1, MK6, TEST and NK cell activity, and high IL-12, MK2, CK1, CK2, CTL, CORT and NPY. Note that several of the cytokines that were once aggregated (IL-1, IL-8, IL-12) now present with differing profiles. These yield a complicated mixed Th1:Th2 profile consistent with our previous analysis of GWI. Further refinement of other aggregate nodes, and inclusion of the Th17 axis is currently underway.
 - *Preliminary alignment with experimental data.* We found in GWI that aligns with the nominal steady state (SS0) at a significance level $p=0.85$ again indicating poor alignment with normal health. Alignment with the alternate steady state (SS1) however was much more significant ($p=0.07$). This both supports a notion of a complex stable combined Th1:Th2 response in this illness, and suggests a brain component in its perpetuation. Further analysis with the refined model is being conducted.

Collectively these simulations of known endocrine-immune circuitry support the existence alternate homeostatic regimes, some of which overlap substantially with observed immune and endocrine status in male GWI and female CFS subjects. Such overlap with naturally occurring stable regulatory regimes would certainly be consistent with the persistence of symptoms long after the initiating event. This same characteristic may also explain why these illnesses appear in many ways resistant to treatment.

4. *An early model of neuroinflammation (extension to Task 3).* Elevated levels of pro-inflammatory cytokines negatively impact learning, memory and neurogenesis. The intense immune activation in the brain that characterizes infections, injury, neurotrauma and severe/chronic stressful conditions, can induce hyper-excitability of neuronal circuits perpetuating an inflammatory state within the CNS resulting in excitotoxicity, and eventually apoptosis and neurodegeneration resulting in learning and memory impairments. To explore these mechanisms we have constructed a first model with Neurons, Neural Progenitor Cells (NPCs), Endothelial Cells (ECs), Microglia, and Astrocytes as key cellular components, while interleukin (IL)-1, IL-4, IL-6, tumor necrosis factor (TNF)- α , and OX-2 membrane glycoprotein (CD200) comprise a simplified neuro-inflammatory response. As numerous studies show communication of inflammatory information to the brain via both humoral and neuronal mechanisms, hormone signaling is included via Insulin-like Growth Factor 1 (IGF-1), Vascular Endothelial Growth Factor (VEGF), Brain Derived

Neurotrophic Factor (BDNF), and cortisol (CORT). The neurotransmission component is conveyed with the inclusion of Acetylcholine (ACh), Norepinephrine (NE), Glutamate (Glut), and Adenosine Triphosphate (ATP). Stress-induced immune activation is a neurally initiated phenomenon, via the activation of noradrenergic pathways and altered cholinergic neurotransmission. Elevated brain cytokines produce further activation of stress response systems such as the HPA axis and the SNS. The multiple feed forward and feedback connections between these elements found in the neurophysiology literature are depicted in the circuit model shown in **Figure 5**.

- Stable immune response modes in the brain - a first analysis: Interaction among these various cell populations via immune, hormone and neurotransmitter signals ultimately revealed two steady states. The first, again, is the normal reference state of health (SS0). The alternate steady state (SS1) is characterized by low levels of ACh, BDNF, IGF-1, IL-4, and VEGF and suppressed activity of Astrocytes, ECells, NPCs, and Neurons, accompanied by elevated levels of CORT, Glut, IL-1, IL-6, and TNF- α , and over activation of Microglia. This is consistent with a chronic neuroinflammatory state. The elevated CORT levels, seen to align with GWI in our other models, suggests a possible involvement of a persistent and stable neuro-inflammatory cascade in this illness.

5. Continued development of treatment design (Task 6, 7). Analysis of the above-mentioned regulatory signaling circuits not only provides information describing the stable steady states available to the system but also extensively describes the ensemble of transitory states that lead *unequivocally* to one steady state or another; these are said to lie within that steady state's *basin of attraction*. Importantly, these subsets of transitory states will lead to that specific stable state independently of an individual's immune and endocrine response kinetics. This guaranteed convergence to a healthy equilibrium makes them attractive as broadly applicable treatment destination states. *In the design of minimally invasive interventions our basic paradigm is therefore to identify the closest transitory state(s) that lie within the basin of attraction that ensures a return to normal homeostasis.*

- A global trajectory search formalism. We have formalized the treatment course as a vector describing a path from the state of disease to health. Allowable transitions between states along the path consist of normal evolution of the system, as described by our logic rules, and transitions induced by clinically feasible interventions. To find treatment course paths that meet these criteria we have formulated our search as a global optimization problem. We have chosen to use a Genetic Algorithm (GA) optimization method, as the discrete nature of our model naturally accommodates the GA solution procedure. Initially, the GA seeds the solution space by generating random solutions composed of binary strings or "chromosomes" representing a treatment path. Each member of this initial population is checked against a fitness function and assigned a fitness score. Top ranking members of the population are then chosen as the parent solutions for the next generation. Each generation is made up of the chosen parent population plus combinations of crossed-over "mated" parent solutions with a small chance for random mutation. This process runs over a set number of generations or until optimum results are found. This allows a rapid search of the global space, while mutations minimize the chance of remaining in local minima.
- A multiple objective criterion. Our fitness function divides the overall the solution string into segments describing each time-step in a treatment course. The overall desirability of a solution is assessed on the basis of three objectives: (i) feasibility or compliance with the model, (ii) compliance with allowable treatment perturbations, and (iii) the minimal invasiveness and duration of treatment. The first and second of these objectives has been implemented in the first trial version. For each time step segment subsequent time-steps are compared to the allowable transition states and assigned a *compliance* score based on the minimum hamming distance separating the proposed solution state and the allowable states. Overall fitness of a solution is then the sum of the hamming measures for all state transitions along a given solution path. A fitness value of zero indicates a perfect compliance with model behavior and allowable interventions.

Based on this paradigm we have started work on **Tasks 6 and 7**. A first fitness function based on model compliance, as well as the GA algorithm itself, have been designed and implemented. Currently, we are in the process of optimizing code parameters (population size, number of generations, mutation rate, etc...) to efficiently search the large state spaces of our multi-systems model.

Additionally, we are currently refining these models as well as the treatment search algorithm to incorporate the effects of timescale. This will make it possible to take advantage of saddle point states or unstable intermediate states that lie between the basins of attraction. We are currently investigating avenues for exploiting broad classes of kinetic scales that might make it possible to reduce the treatment complexity even further and tailor these interventions to patient sub-groups.

6. Continuing work. Ongoing work involves the continued refinement of a circuit model describing mechanisms of neuroinflammation and neurotransmission in the brain. Efforts are also now shifting to the completion and validation of the treatment design algorithm. This will be the major area of development as we begin simulation of treatment strategies, the principal deliverable of this project.

Timeline. As described in the previous report dated September 30, 2011, the University of Alberta's Research services Office submitted on behalf of the principal investigator a request for a one-year extension of the project term due to administrative delays. This request was reviewed initially by Ms. Strock and Dr. Phillips of the DoD (January 23, 2012) and we were asked to resubmit this request at a later date (6-8 months before end of project term). We have since confirmed with Dr. Rebecca Fisher that this continues to be the correct course of action (ref. email from Dr. Fisher dated September 21, 2012). In accordance with Dr. Fisher's recommendation we are submitting a formal request for a one-year no-cost extension as part of our request to transfer this award to Nova Southeastern University retroactive to June 1, 2013.

Key Research Accomplishments

In keeping with the milestones described in the project submission initial efforts were directed at:

- Consistent with the previously completed Task 1, we have further improved the efficiency of the serial C code, again delivering order of magnitude improvements in execution speed and memory usage. Importantly we have engineered a parallel framework based on MPI and Pthread protocols to deploy this code onto distributed high-performance platforms. This code is now deployed and fully operational on the University of Miami Pegasus 2 platform.
- Consistent with the now completed Task 2, we have continued to refine our previous model of immune signaling mechanisms. These now include the actions of Th3 and Th17 axes, implemented in models at two levels of granularity.
- We have now basically completed Task 3 as defined originally. In this regard we have produced a refined multi-axis model, further developed our validation scheme and submitted to PLoS One a first complete manuscript describing co-regulation across HPA, HPG and immune axes in men and women.
- In an extension to original Task 2 and 3, we have produced a first circuit model of inflammatory processes occurring in the brain and involving the cell types and immune signaling specific to this physiological compartment. Early analyses of this model show the persistence of a chronic neuroinflammatory state perpetuated by overactive microglia and underactive astrocytes, leading to loss of neuron function, in conjunction with elevated levels of cortisol.
- Consistent with Task 4 and Task 5 we have conducted a refined analysis of multi-stability properties of both the broad HPA-HPG-immune model and the detailed BIS immune model. Comparing predicted equilibrium states with experimental immune and endocrine data from male and female GWI and CFS subjects we find:
 - Male GWI and CFS subjects align with states showing hypercortisolism, low testosterone, elevated Th1 inflammatory cytokines, decreased NK cell activity in conjunction with an

elevated Th17 response, although male CFS subjects also show a propensity to align with an elevated Treg response suggesting a mixed immune signature for this illness not seen in GWI.

- Female CFS subjects align with states showing hypocortisolism, elevated estrogen, and a shift towards Th2 activation.
- We have re-assessed our approach to treatment design (Task 6, 7) and have begun encoding an approach based on a global search for a treatment course assisting an optimal walk through a discrete endocrine immune state space leading from an illness to a healthy condition.

Reportable Outcomes.

The results of these latest analyses are being communicated as follows:

- The previous draft manuscript Craddock et al., 2013, enclosed as Appendix A, has been extensively revised and is now submitted to the journal PLoS One. Similarly we expect the extensions and revisions to the detailed immune model (working document in Appendix B, Annual Report 2012, Fritsch et al., 2013), to be ready for submission to the journal Molecular Systems Biology by October this year.
- Early results were presented at a closed meeting sponsored by the CDC and the CFIDS Association of America and held at the Cold Spring Harbor Laboratory's Banbury Centre in Long Island, NY (Sep. 30 -Oct 3, 2012).
- We will be submitting two abstracts for oral presentation at the IACFS/ME 11th Biennial International Research and Clinical Conference to be held in San Francisco, California, USA, March 20-23, 2014. The conference is co-sponsored by Stanford University.

Regarding synergy with complementary research efforts, these findings were recently used to secure an invited GWIRP Consortium Award, now awarded (prime institution - Nova Southeastern University). The are also being used in support of 2 VA Merit applications that have been reviewed and invited for resubmission this month.

Conclusions.

We are currently processing a formal request for a one-year no-cost extension of the project term due to a delayed start. We have carried out a major shift in paradigm and continue to refine these regulatory circuit models as well as developing new components such as the neuroinflammatory model. The basic algorithmic framework has now been translated and re-engineered to deploy larger more detailed models on distributed high-performance platforms like the Pegasus 2 platform at the University of Miami.

Simulations based on these models have shown that the illness-specific effects of gender are particularly striking. Work continues on the refinement of the intervention design component. Initial analyses favor the deployment of a joint hormone-immune intervention over strategies that target these systems separately.

Personnel receiving support from this award:

- Travis Craddock, Ph.D., Senior Research Associate, Broderick Clinical Systems Biology Laboratory, University of Alberta;
Now co-investigator and Assistant Professor of Psychology, Nova Southeastern University, FL.
- Lundy McKibbin, undergraduate student MD class of 2017, Undergraduate Research Intern, Broderick Clinical Systems Biology Laboratory, University of Alberta;
- Simar Singh, M.Sc. graduate 2012, Graduate Research Intern, Broderick Clinical Systems Biology Laboratory, Nova Southeastern University, Institute for Neuro-immune Medicine
- Mark Rice, undergraduate student computer science, Research Intern and Chief Programmer, Nova Southeastern University, Institute for Neuro-immune Medicine; Clinical Systems Biology Group (Broderick and Craddock)

- Ryan del Rosario, undergraduate student MD class of 2017, Research Intern and Lead HPC Programmer, Nova Southeastern University, Institute for Neuro-immune Medicine; Clinical Systems Biology Group (Broderick and Craddock).

References.

1. Thomas R, Thieffry D, Kaufman M (1995) Dynamical behaviour of biological regulatory networks--I. Biological role of feedback loops and practical use of the concept of the loop-characteristic state. *Bull Math Biol.* 57: 247-276.
2. Thomas R (1991) Regulatory Networks Seen as Asynchronous Automata: A Logical Description. *J Theor Biol* 153: 1-23.
3. Mendoza L, Xenarios I (2006) A method for the generation of standardized qualitative dynamical systems of regulatory networks *Theor Biol Med Model* 3: 13.
4. Ben-Zvi A, Vernon SD, Broderick G. (2008). Model-based Therapeutic Correction of Hypothalamic Pituitary Adrenal Axis Dysfunction. *PLoS Comput Biol* 5(1): e1000273. doi:10.1371/journal.pcbi.1000273.
5. Brown M (1975). A method for combining non-independent, one-sided tests of significance. *Biometrics* 31: 987–992.
6. Craddock TJA, Miller DB, Fletcher MA, Klimas NG, Broderick G. (2013). Towards an Integrative Model of Complex Stress-Mediated Illnesses: Gulf War Illness and Chronic Fatigue Syndrome. *PLoS One, Submitted*.
7. Broderick G, Kreitz A, Fuite J, Fletcher MA, Vernon SD, Klimas N (2011). A pilot study of immune network remodeling under challenge in Gulf War Illness. *Brain Behav Immun.* Feb; 25(2): 302-13.
8. Broderick G, Fuite J, Kreitz A, Vernon SD, Klimas N, Fletcher MA (2010). Formal Analysis of Cytokine Networks in Chronic Fatigue Syndrome. *Brain Behav Immun*, Oct; 24(7): 1209-17.
9. Fuite J, Vernon SD, Broderick G (2008). Neuroendocrine and Immune Network Re-modeling in Chronic Fatigue Syndrome: An Exploratory Analysis. Invited submission, *Genomics*; 92(6): 393-399.
10. Folcik VA, An GC, Orosz CG (2007). The Basic Immune Simulator: an agent-based model to study the interactions between innate and adaptive immunity. *Theor Biol Med Model.* Sep 27;4:39.
11. Folcik VA, Broderick G, Mohan S, Block B, Ekbote C, Doolittle J, Khoury M, Davis L, Marsh CB (2011). Using an agent-based model to analyze the dynamic communication network of the immune response. *Theor Biol Med Model.* Jan 19;8:1.
12. Fritsch P, Craddock TJA, Smylie AL, Folcik Nivar VA, Fletcher MA, Klimas NG, de Vries G, Broderick G (2012). A Study of Multiple Homeostatic Regimes in a Discrete Logic Model of Immune Signaling. In preparation.
13. Broderick G, Ben-Hamo R, Vashishtha S, Efroni S, Nathanson L, Barnes Z, Fletcher MA, Klimas N. (2013). Altered immune pathway activity under exercise challenge in Gulf War Illness: an exploratory analysis. *Brain Behav Immun.* Feb;28:159-69.
14. Fletcher MA, Rosenthal M, Antoni M, Ironson G, Zeng XR, Barnes Z, Harvey JM, Hurwitz B, Levis S, Broderick G, Klimas NG. (2010). Plasma neuropeptide Y: a biomarker for symptom severity in chronic fatigue syndrome. *Behav Brain Funct.* Dec 29;6:76.

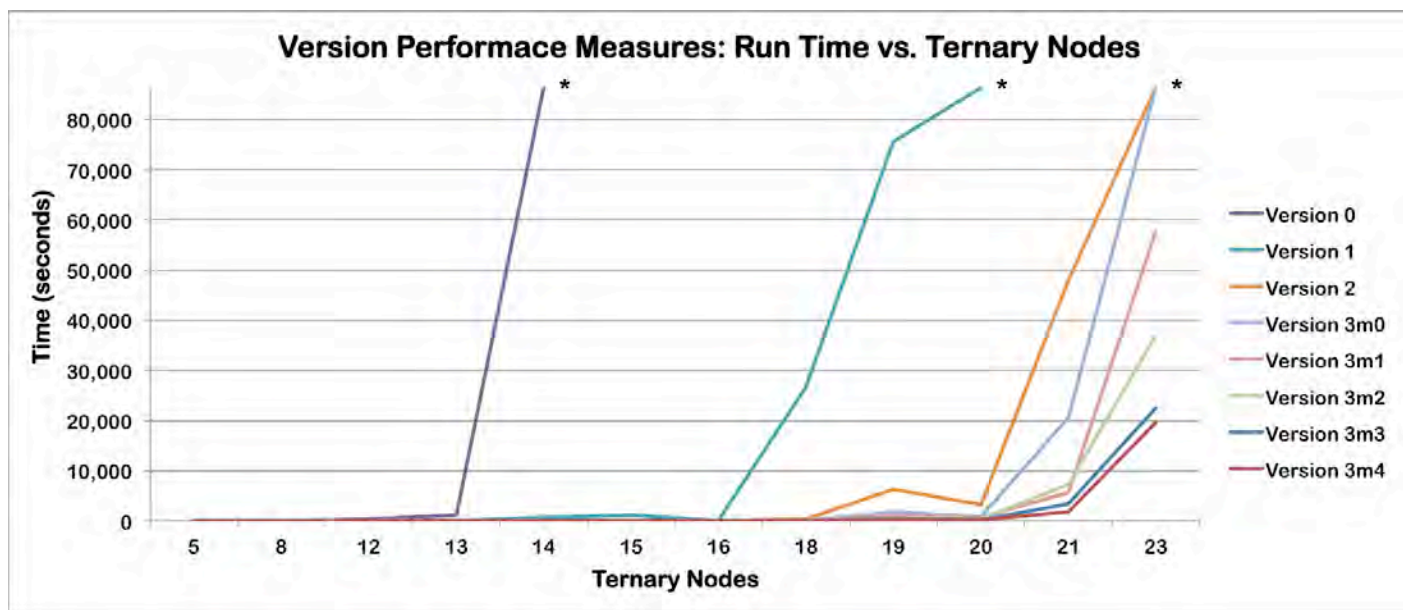


Figure 1. *Evolution of computational performance.* Evolution of computer wall time as a function model complexity described in terms of the number of state variables (ternary nodes). In less than a year, re-engineering of the computer code supporting the identification of stable states in a regulatory system has enable an almost 2-fold increase in the number of state variables in the circuit model.

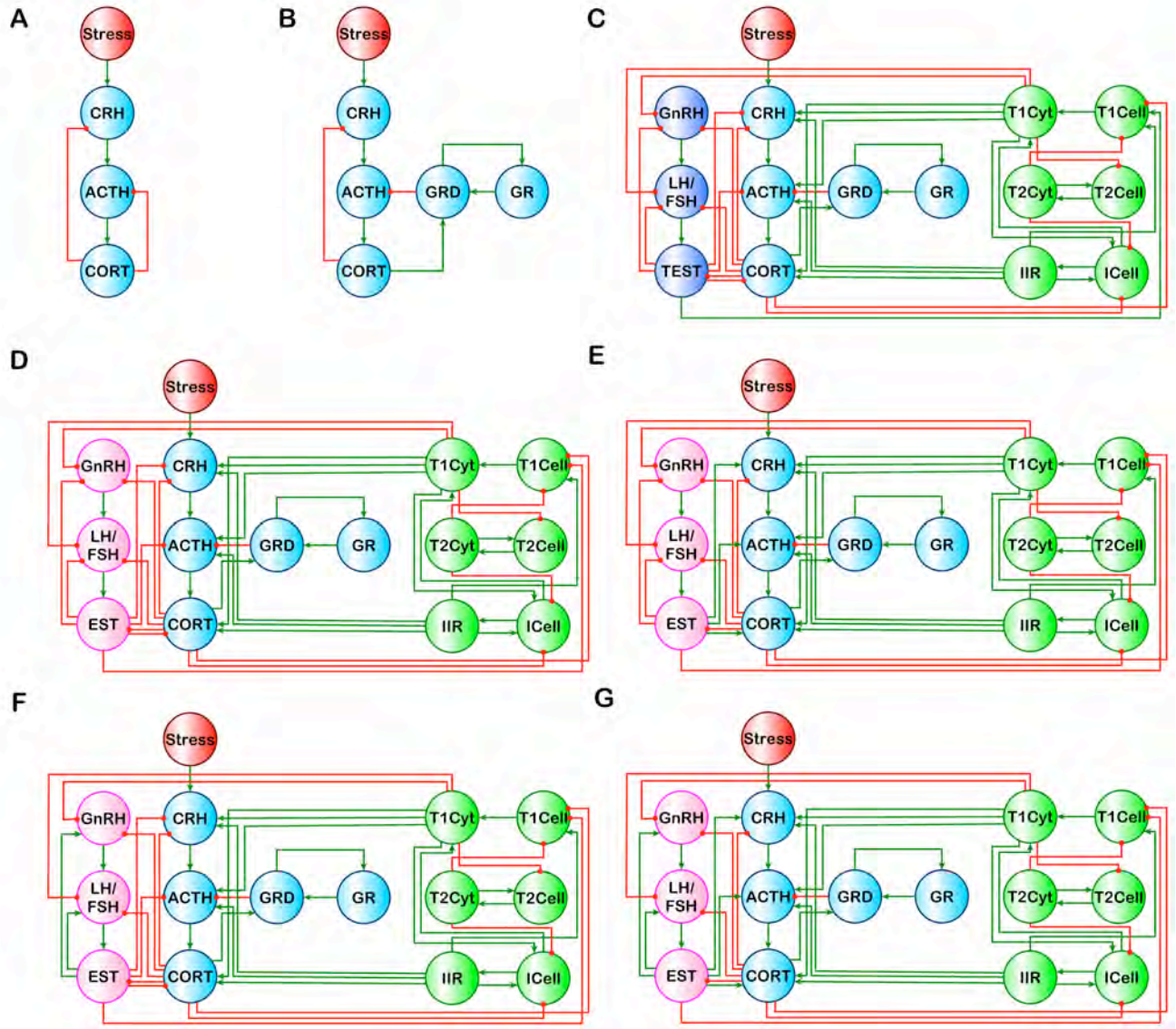


Figure 2. *Increased granularity multi-axis model.* A significant revision of the discrete circuit model of HPA function (A,B) augmented with HPG-immune interactions in male subjects (C) and female subjects (D-G) in the specific case of positive feedback along the female HPG axis and suppressive interaction with the HPA axis (revised and resubmitted manuscript) [6]. Green directed edges represent an up-regulation of the target by the source node whereas a red terminal edge represents a suppressive action.

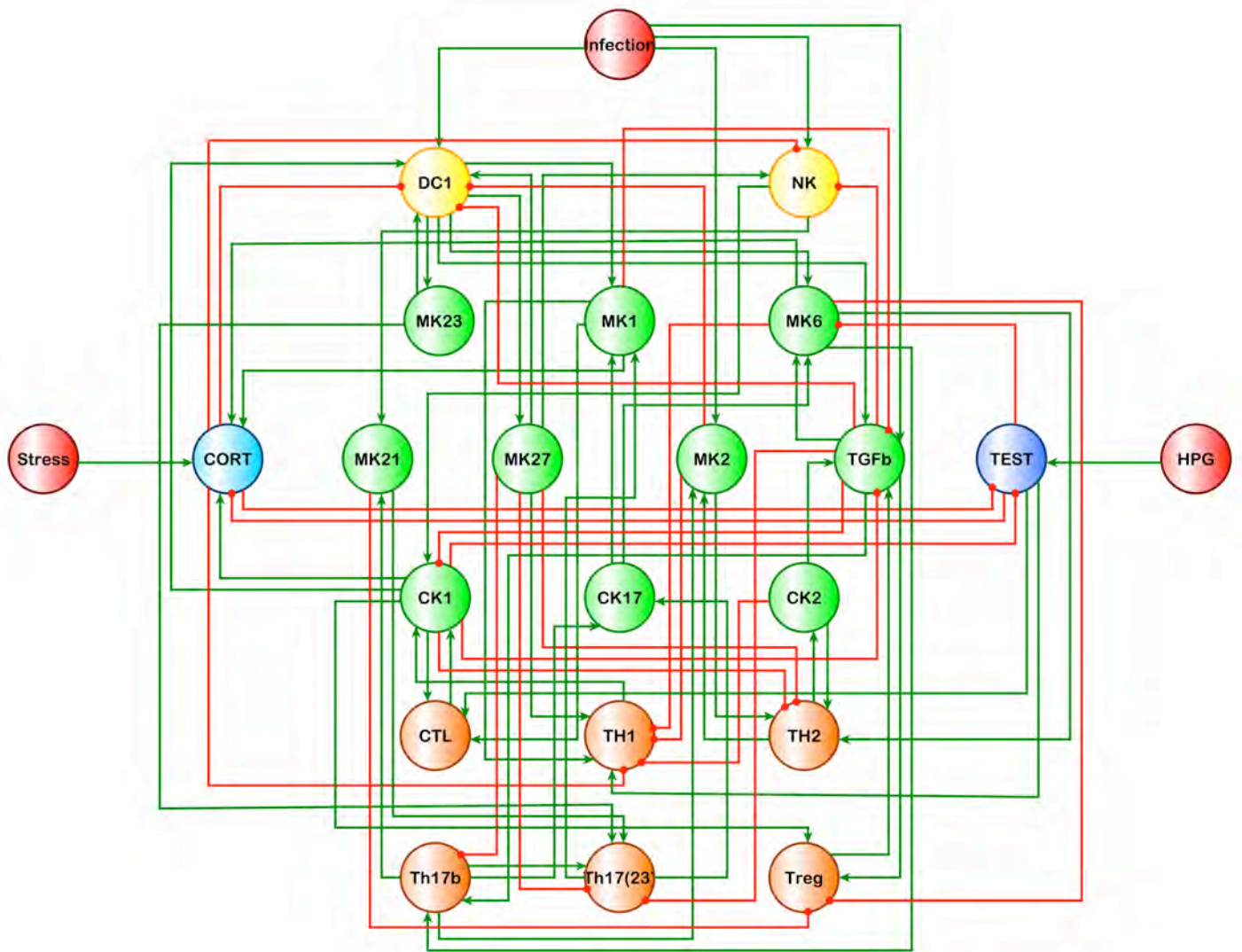


Figure 3. Detailed Immune model revised. Circuit diagram of the detailed immune system model revised to include elements of Th17 and Treg activity mediated by TGF- β , IL-21, IL-23, IL-27 and others. This is a significant increase in granularity from the previous such model and has resulted in a revision of draft manuscript, now underway and due for submission before year end [12].

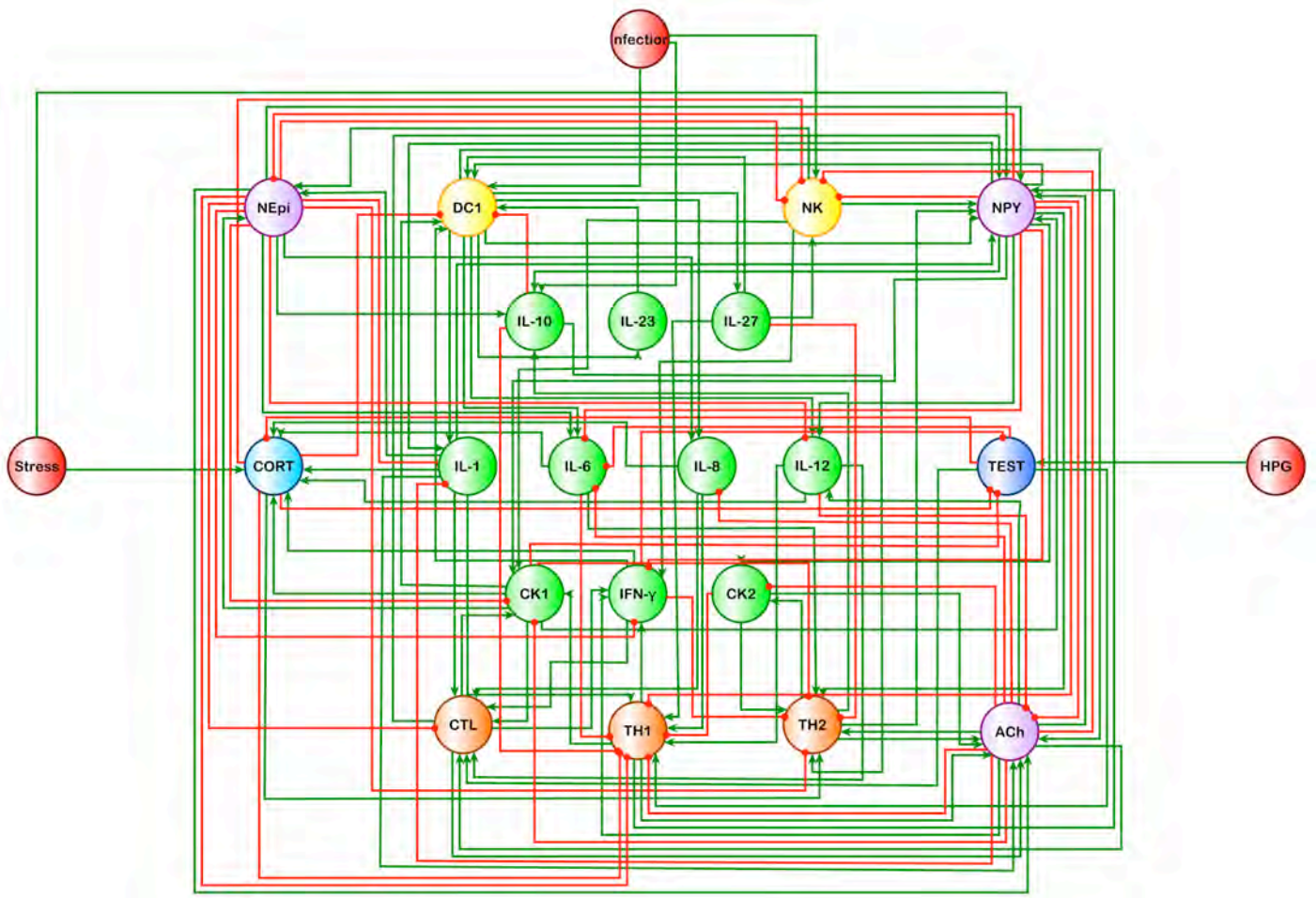


Figure 4. *High-resolution immune model with neurotransmission oversight.* Circuit diagram of a first prototype model capturing fine-grained immune signaling with the contribution of immune modulating neurotransmitters neuro-peptide Y (NPY), acetylcholine (ACh) and norepinephrine (NEpi). This model is still in progress and will incorporate the Th17 and Treg axes as well as additional neurotransmitters as we move forward.

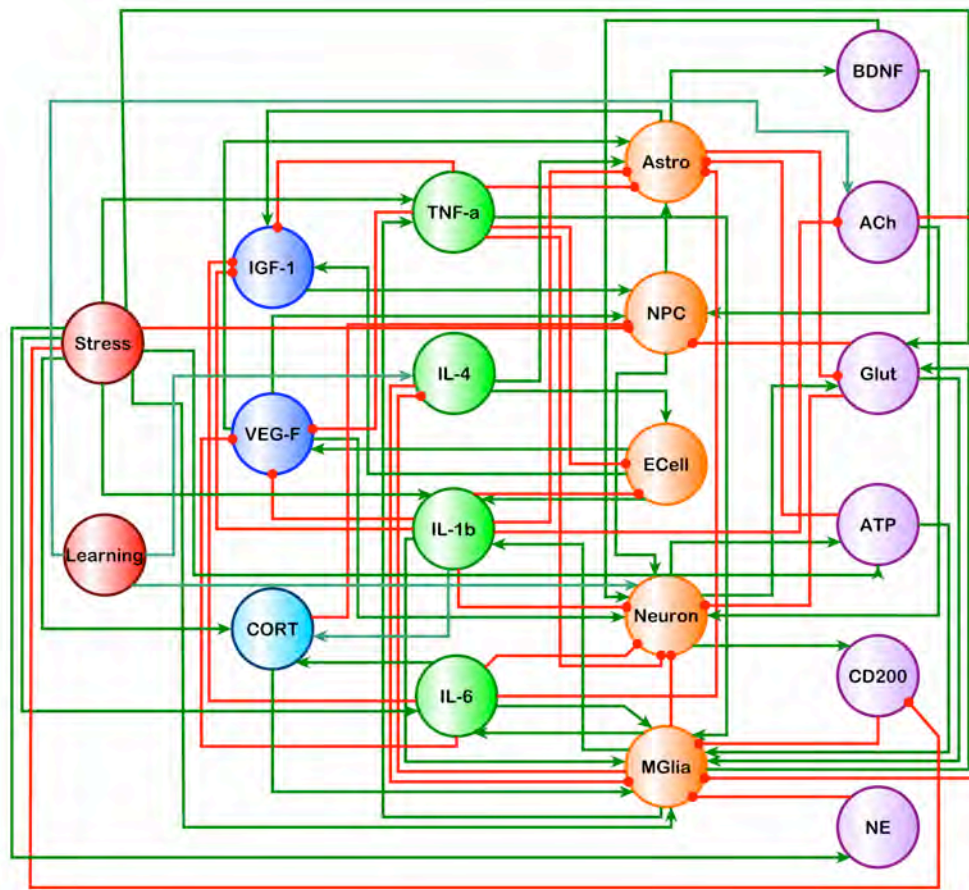


Figure 5. *A first prototype model of brain immunity.* A first circuit model describing the regulation of neuro-inflammation in the brain that includes the role of neurons, neural progenitor cells (NPCs), endothelial cells (ECells), microglia, and astrocytes as key cellular components as well as basic signaling mechanisms involving interleukin (IL)-1, IL-4, IL-6, tumor necrosis factor (TNF)- α , and OX-2 membrane glycoprotein (CD200) and other molecular messengers.

Appendix A:

Craddock TJA, Fritsch P, Rice MA Jr, del Rosario RM, Miller DB, Fletcher MA, Klimas NG, Broderick G. A Role for Homeostatic Drive in the Perpetuation of Complex Chronic Illness: Gulf War Illness and Chronic Fatigue Syndrome. 2013, *Submitted PLoS One, Under editorial review*.

A Role for Homeostatic Drive in the Perpetuation of Complex Chronic Illness: Gulf War Illness and Chronic Fatigue Syndrome

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Abstract

A key component in the body's stress response, the hypothalamic-pituitary-adrenal (HPA) axis orchestrates changes across a broad range of major biological systems. Its dysfunction has been associated with numerous chronic diseases including Gulf War Illness (GWI) and chronic fatigue syndrome (CFS). Though tightly coupled with other components of endocrine and immune function, few models of HPA function account for these interactions. Here we extend conventional models of HPA function by including feed-forward and feedback interaction with sex hormone regulation and immune response. We use this multi-axis model to explore the role of homeostatic regulation in perpetuating chronic conditions, specifically GWI and CFS. An important obstacle in building these models remains the scarcity of *in vivo* kinetic data. We circumvented this using a discrete logic representation based solely on literature of physiological and biochemical connectivity to provide a qualitative description of system behavior. This connectivity model linked molecular variables across the HPA axis, hypothalamic-pituitary-gonadal (HPG) axis in men and women, as well as a simple immune network. Inclusion of these interactions produced at multiple alternate homeostatic states. Experimental data for endocrine-immune markers measured in male GWI subjects showed the greatest alignment with predictions of a naturally occurring alternate steady state presenting with hypercortisolism, low testosterone and a shift towards a Th1 immune response. In female CFS subjects, expression of these markers aligned with an alternate homeostatic state displaying hypocortisolism, high estradiol, and a shift towards an anti-inflammatory Th2 activation. These results support a role for homeostatic drive in perpetuating dysfunctional cortisol levels through persistent interaction with the immune system and HPG axis. This same basic drive may also perpetuate sexually dimorphic responses due to inherently different behavior of the male and female HPG. Though coarse, these models may nonetheless support the design of robust treatments that might exploit these regulatory regimes.

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis, a key component in the body's stress response, serves to articulate changes in a broad range of homeostatic regulators as a function of environmental cues. Such cues can consist of both physical stressors (injury, infection, thermal exposure) and psycho-emotional stressors (frustration, fear, fight or flight decisions). Instantiation of this survival program is accomplished through controlled modulation of the neuroendocrine and immune systems, as well as the sympathetic nervous systems [1-3]. Considering its function as a broad-reaching integrator of major physiological systems, it is no surprise that numerous chronic conditions have been associated with abnormal regulation of the HPA axis, including major depressive disorder (MDD) [4, 5], post-traumatic stress disorder (PTSD) [6-8], Alzheimer's disease [9], Gulf War Illness (GWI) [10-12], and chronic fatigue syndrome (CFS) [13-15]. When compared to non-deployed veterans, Golier et al. [10] found that symptomatic Gulf War veterans without psychiatric illness, as well as veterans with PTSD alone, showed significantly greater cortisol suppression to dexamethasone (DEX) suggesting markedly enhanced negative feedback along the HPA axis. Further study by these same investigators indicated that this might be due to a significantly attenuated ACTH response by the pituitary in veterans with GWI without PTSD [11, 12]. A similar suppression of cortisol response to DEX was found in CFS subjects by Van Den Eede et al. [13] with this being further exacerbated by oestrogen intake. With regard to HPA circadian dynamics, CFS subjects were found to exhibit significantly increased adrenal sensitivity to ACTH and marginally increased inhibitory feedback during the nocturnal period when compared with control subjects and CFS subjects comorbid with fibromyalgia (FM) [14, 15]. Conversely the pain-dominant CFS-FM subjects showed significantly blunted cortisol inhibitory feedback. While evidence such as this implicates abnormal regulation of HPA function leading to chronic hypocortisolic and hypercortisolic states in these illnesses, the genesis of this dysregulation is unclear.

Previously we investigated the possibility that some of these pathological states may coincide with naturally occurring alternate homeostatic stable states [16]. These “backup programs” would offer a way of maintaining homeostatic control in crisis situations at the cost of reduced function. The existence of such multiple stable states is characteristic of systems that incorporate feed-forward and feedback mechanisms. Feedforward loops in biology play the crucial role of driving rapid acute responses, while feedback loops will generally limit the extent of a response. Both will also drive complex dynamic behavior, including differentiation and periodicity [17]. While small perturbations may force temporary departures, these systems return to their original resting states once these perturbations are removed. If however, the perturbation is of significant strength and duration, the system may be incapable of returning to its normal operating regime and instead may assume a new alternate resting state. Knowledge of the system dynamics can allow us to map these different stable states and several mathematical models of the HPA exist [18-26]. So far, only one such model is known to accommodate multi-stability in the dynamic behavior of the HPA axis. It does so via the addition of a feed-forward mechanism involving dimerization of the glucocorticoid receptor (GR) complex [27] (Figure 1). In this process glucocorticoid (GC) bound GRs form homodimers that translocate into the cell nucleus to bind DNA, up-regulating GR synthesis and producing a positive feedback loop. However, this model and the majority of other models do not extend beyond the physiological boundaries of the HPA axis itself and thus are limited in their predictive capabilities. As discussed in the following sections, HPA activity is intertwined with the behavior of the hypothalamic-pituitary-gonadal (HPG) axis and the immune system, among others, and this interplay should not be ignored when considering the number and nature of stationary states available to the overarching system. Our hypothesis is that these alternate regulatory regimes may facilitate the persistence of complex chronic illnesses like GWI and CFS. To evaluate the role of alternate homeostatic attractors in these illnesses we constructed a computational model of regulatory control linking the HPA, HPG and immune systems.

There is a substantial body of physiological and biochemical data for many biological systems describing the connectivity between molecular and cellular elements, the presence of recurring structural motifs and functional modules. For example, negative autoregulation, in which a transcription factor represses its own transcription, is a simple network motif observed in many transcription networks. While, numerous motifs have been found in biological networks (negative/positive autoregulation, coherent/incoherent and multi-output feedforward loops, single-input modules and dense overlapping regulons) [28], data regarding the precise stoichiometry and kinetics of these systems in humans is extremely limited. Many existing models rely heavily on animal data as a source of kinetic parameters, or adopt general order of magnitude estimates when this data is lacking. To circumvent this issue and draw on the rich body of known molecular and cellular interactions in physiological and biochemistry, we have adopted the discrete logical network methodology proposed originally by Thomas et al. [29, 30] and developed further by Mendoza and Xenarios [31]. By applying logic rules to a network of known interactions it is possible to identify the number of stable resting states, their type as well as their molecular and cellular description, without detailed knowledge of the response dynamics. In this work we use this method to extend our previous analysis of human HPA axis dynamics by including its regulatory interactions with the neighboring HPG axis and immune system. This resulting mathematical model better represents the complexity of endocrine-immune interactions by supporting the detection and identification of alternate resting modes of the HPA-HPG-immune axis. Based on connectivity information alone, we show that multi-stability is easily obtained from these interacting systems. Moreover, we show that experimental data from our on-going studies of GWI and CFS show better alignment with these alternate resting modes than with the typical healthy homeostatic stable state. Ultimately, knowledge of such homeostatic modes could be used to identify promising applications of pharmaceutical, hormone and/or immune therapy that exploit the body's natural dynamics to reinforce treatment effects.

Methods

Ethics Statement

All subjects signed an informed consent approved by the Institutional Review Board of the University of Miami. Ethics review and approval for data analysis was also obtained by the IRB of the University of Alberta.

An Integrative Multi-systems Model of the HPA-HPG-Immune System

There is a substantial amount of physiological data describing the HPA, HPG and immune systems as stand-alone entities. To a much lesser degree there also exists evidence for the mutual interactions between these systems. The following sections describe the experimental evidence used to infer the topology of an overarching HPA-HPG-immune interaction network (Figure 1).

The HPA Axis: Activation of the HPA axis begins at the paraventricular nucleus (PVN) of the hypothalamus. Specifically, afferents transmitting stress related information in the brain converge on the medial parvocellular neurons of the PVN inducing the release of several peptides, including corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), into the pituitary hypophyseal-portal circulation. The unique vascular system allows very small quantities of these hypothalamic hormones to act directly on their targets in the anterior pituitary without dilution by systemic circulation. CRH and AVP act in conjunction on membrane bound CRH-R1 receptors in the anterior pituitary to stimulate adrenocorticotrophic hormone (ACTH) synthesis, and its rapid release into peripheral circulation. ACTH circulates to the adrenal cortex where it acts on the membrane bound MC2-R receptor to stimulate the release of GCs (corticosterone in the rat, and cortisol (CORT) in humans and nonhuman primates). To regulate the stress response, GCs exert negative feedback at the hypothalamus and pituitary to inhibit further synthesis and release of CRH and ACTH, respectively [32]. This is the standard view of the HPA axis utilized in the majority of models (Figure 1 A). However, as noted by Gupta et al. [27] circulating glucocorticoids act via

cytosolic GRs, which, unlike membrane bound receptors, dimerize (GRD) and translocate into the cell nucleus upon activation to up-regulate GR synthesis and interact with other relevant transcription factors, or GC-sensitive genes (Figure 1 B). Gupta et al. included this GR expression feedforward loop at the pituitary, as it is a main driver of the HPA axis, and found a resulting bistability in the HPA system [27]. However, all nucleated cells possess GRs, as GCs influence practically every system in the body, suggesting this feedforward loop may be important in other tissues beyond the HPA axis. As described below major systems affected by GCs include the HPG axis and immune system.

The HPG Axis: GCs have an inhibitory effect on the HPG axis, a central regulator of the reproductive system, at all levels [33-37]. Activation of the HPG starts from brain generated pulsatile signals that stimulate the preoptic area of the hypothalamus to produce gonadotropin-releasing hormone (GnRH). GnRH is secreted into the pituitary hypophyseal portal bloodstream, which carries it to the pituitary gland, where it activates membrane bound GnRH-R receptors, resulting in the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into circulation. These gonadotropins flow to the gonads where they work synergistically to promote the secretion of the sex steroids. In males, LH binds to receptors on Leydig cells in the testes to stimulate the synthesis and secretion of testosterone (TEST). In females, LH activates receptors on Theca interna cells in the ovaries to stimulate the release of androstenedione, which is aromatized by granulosa cells to produce estradiol (EST), and progesterone (PROG). TEST negatively feeds back on the HPG to inhibit GnRH, FSH and LH secretion and synthesis [33]. This feedback mechanism is somewhat more complex in females where, depending on the phase of the female menstrual cycle, EST and PROG can exert either positive or negative feedback on the production and release of GnRH and the gonadotropins [36, 38, 39].

A lesser-known aspect is that several components of the HPG axis exert reciprocal effects on the HPA axis [33, 34, 36]. Testosterone exhibits an inhibitory effect on all levels of the

HPA [33] (Figure 1 C), whereas EST and PROG can serve to stimulate or inhibit the HPA axis depending on menstrual cycle phase, or phase of life [34]. These affects may be mediated through changes in adrenocorticoid synthesis, stress-induced ACTH and GC release, and CRH and AVP synthesis in the PVN, by direct activation of oestrogen and androgen receptors along the HPA or via interaction between GRs and sex steroid receptors to regulate transcription [33,34,36]. Thus, an interactive functional crosstalk exists between the HPA and HPG axes, which cannot be ignored when investigating HPA axis regulation and dysfunction. Mutual inhibition between the HPA and HPG (Figure 1 C) was considered standard for males. However, as it is not clear whether the EST and PROG inhibition/stimulation of the HPA occurs in coordination with the inhibition/stimulation of the HPG, these cases were explored for females alone as separate alternative models of the HPA-HPG interaction (Figure 1 D-G) in addition to the model considered for males.

A Simple Model of the Immune System: While not typically considered part of the neuroendocrine system, the immune system plays a very important role in regulating the HPA axis. Here we base our simplified immune system upon our previous work detailing the communication network of the immune response [40]. Cells of the innate immune response (ICells), including mononuclear phagocytes, such as macrophages, and dendritic cells, natural-killer (NK) cells, endothelial cells and mucosal epithelial cells, communicate via the release of numerous cytokines. Cytokines that regulate the innate immune response (IIR) include interleukin (IL) -1, IL-6, IL-8 and tumor necrosis factor alpha (TNF- α), and can also include IL-12, a primary mediator of early innate immunity. Primarily, these signals serve to activate and recruit other ICells, which in turn produce more cytokines. IL-15, which stimulates proliferation of NK cells and effector T-lymphocytes, can also be considered as part of the IIR as well as IL-23, an important inflammatory signal contributing to the Th17 response against infection.

IIR signals can also serve to prime helper T cells towards a Th1 type adaptive immune response (T1Cell). This response produces Th1 proinflammatory cytokines (T1Cyt) including IL-2, interferon-gamma (IFN- γ), and tumor necrosis factor beta (TNF- β), which further activates ICells, while suppressing the Th2 adaptive immune response (T2Cell). The T2Cell is responsible for the production of the Th2 anti-inflammatory cytokines (T2Cyt) IL-4, IL-5, IL-10 and IL-13, which have important anti-inflammatory and immunosuppressive activities, and serve to inhibit the activity of T1Cell and ICells.

Cytokines can also serve as mediators between the immune and endocrine systems. Between the HPA and the immune network there exists a mutual crosstalk [41-43] (Figure 1 C-G). The IIR and T1Cell cytokines selected here serve to stimulate the HPA axis at all levels [41-43]. CORT, in turn, acts to suppress the activity of ICells (specifically NK cells [44], and DC cells [45]), and the T1Cell [46] causing a shift from the inflammatory to the anti-inflammatory response [41, 42, 47]. The interaction between the HPG and the immune system is complex and sexually dimorphic, and is still an active field of research. However, at a general coarse level of description TEST serves to stimulate the development of the Th1 response [48] (Figure 1 C), whereas EST inhibits the Th1 response causing a shift towards the Th2 anti-inflammatory response [48,49]. The reciprocal crosstalk from the immune system to the HPG is equally intricate. In broad terms this conversation is communicated via T1Cyt. Receptors for TNF- α and IFN- γ are expressed in testicular Leydig cells and there is evidence that these cytokines can directly inhibit testosterone production [50]. TNF α also decreases the release of GnRH in the hypothalamus and LH in the pituitary gland in both males [50] and females [51] eventually leading to a decrease in sex steroid levels. As such, we model the T1Cyt as inhibiting GnRH and LH/FSH in both male and female models.

A Discrete State Representation

Following the methods of Thomas et al. [29, 30], and more recently Mendoza and Xenarios [31], the neuroendocrine-immune system was represented as a connectivity model consisting of interconnected molecular and cellular variables with three discrete states: -1 (inhibited), 0 (nominal) and 1 (activated). According to this type of model the current state of all variables in a system is described by a state vector $\vec{x}(t)$, such that:

$$x_t = x_1 t, x_2 t, \dots, x_N t \quad (1)$$

where $x_N(t)$ is the state of the N^{th} variable of the system at time t . The image vector $\vec{x}(t+1)$ describes the preferred state towards which the system evolves in the next time increment. The state value of the image vector for the i^{th} variable is determined from its current state and a set of balanced ternary logic statements based on the current value of variable and the mode of action (i.e. activate or inhibit) of the neighboring input variables. These logic statements are expressed as follows (Eq. 2):

$$x_{it+1} = (x_{i1} A t \vee x_{i2} A t \dots x_{ij} A t) \nabla (x_{i1} I t \vee x_{i2} I t \dots x_{ik} I t) (x_{i1} A t \vee x_{i2} A t \dots x_{ij} A t) \neg (x_{i1} I t \vee x_{i2} I t \dots x_{ik} I t) \quad (2)$$

where the ∇ , \vee , and \neg symbols are ternary HIGH/LOW PASS, OR and NOT operators, x_{ij}^A is the state of the i^{th} variable's j^{th} activator, x_{ik}^I is the state of the i^{th} variable's k^{th} inhibitor. The ternary operators given in Equation (2) are described in further detail in Supplementary Tables 1- 3. The first entry in Equation (2) is used when the variable possesses both activators and inhibitors, the middle when the variable has only activators and last when the activator has only inhibitors.

Applying Equation (2) to each variable in the model for the m^{th} state of the system, $\vec{x}^m(t)$, defines the image vector $\vec{x}^m(t+1)$ for that state. With $\vec{x}^m(t+1)$ defined, the system may be updated asynchronously (allowing only one variable to change at a time) following the

generalized logical analysis of Thomas et al. [29, 30]. According to this method the i^{th} variable of the m^{th} state vector $\vec{x}^m(t)$ is moved one step towards its preferred image $\vec{x}^m(t+1)$ (e.g. If $\vec{x}^m(t) = -1$ and $\vec{x}^m(t+1) = 1$, then $\vec{x}_i(t)$ is set to 0). Thus, for each current state of the system there are potentially several subsequent states towards which it may asynchronously evolve.

The number of states, and the values they can be assigned, determine the total number of states available to the model system. With the ternary logic used here, a model of N variables possesses 3^N states. As a result, the number of states increases rapidly as new variables are added. By analyzing all possible states of the system a temporal sequence of states may be discerned. To interpret the results, each state of the system can be represented as an element in a graph. The evolution from one state to a subsequent state can be represented as a directed edge between the two states in this graph. Representation of the state trajectories in this fashion makes it possible to draw on the concepts and tools of graph theory for analysis of the system dynamics. Steady states are defined as those states for which the image vector is the same as the current state vector; in other words the state possesses an out degree of 0.

Comparison to Model

GWl Cohort Sample Collection: Similar cytokine profiles and endocrine measures were obtained as part of a larger ongoing study of 27 GWl and 29 HC subjects recruited from the Miami Veterans Administration Medical Center. Subjects were male with an average age of 43 years and BMI of 28. Inclusion criteria was derived from Fukuda et al. [52], and consisted in identifying veterans deployed to the theater of operations between August 8, 1990 and July 31, 1991, with one or more symptoms present after 6 months from at least 2 of the following: fatigue; mood and cognitive complaints; and musculoskeletal complaints. Subjects

were in good health prior to 1990, and had no current exclusionary diagnoses [53]. Use of the Fukuda definition in GWI is supported by Collins et al. [54]. Control subjects consisted of gulf war era sedentary veterans and were matched to GWI subjects by age, body mass index (BMI) and ethnicity. Additional details regarding this cohort and the laboratory assays performed are available in Broderick et al. [55].

CFS Cohort Sample Collection: Levels of cortisol (CORT) and estradiol (EST) measured in peripheral blood were obtained from the Wichita Clinical dataset [56] for a group of 39 female CFS subjects and 37 Healthy controls (HCs) with an average age of 52 years and an average body mass index (BMI) of 29. Additional details of this cohort and the laboratory assays performed may be found in work previously reported by our group [57, 58]. Multiplex cytokine profiles were obtained in plasma from a separate but demographically comparable cohort of 40 female CFS subjects and a group of 59 healthy female matched control subjects studied by our group at the University of Miami [59]. Average age in this cohort was 53 years with an average BMI of 26. Profiling of cytokine concentrations was performed in morning blood plasma samples using an enzyme-linked immuno-absorbent assay (ELISA)-based assay. Details of this protocol and results of a comparative analysis of cytokine expression patterns are available in Broderick et al. [59]. In both studies a diagnosis of CFS was made using the International Case Definition [53,60]. Exclusion criteria for CFS included all of those listed in the current Centers for Disease Control (CDC) CFS case definition, as well as psychiatric exclusions, as clarified in the International CFS Working Group [60].

Statistical Analysis: Brown's theoretical approximation [61] of Fisher's statistics was used to calculate the significance of alignment between experimental data and a given model predicted state . Fisher's method, a meta-analysis technique, combines probabilities to obtain the overall significance of a set of *P*-values obtained from independent tests of the same null hypothesis. The combined χ^2 statistic,

$$T_0 = -2 \sum_{i=1}^N \ln(p_i) \quad (3)$$

where N is the number of measurable variables and p_i is the corresponding P -values under the null hypothesis, has a χ^2 distribution with $2N$ degrees of freedom assuming that the performed tests are independent. As the molecular variables of the endocrine and immune system interact with one another, as evidenced by the above connectivity diagrams, they are not independent. As a result, direct application of this test statistic is invalid, since the assumption of independence is violated. Brown [61] suggested a method for combining non-independent tests. If the tests are not independent, then the statistic T_0 has mean $m = 2N$ and variance (σ^2) given as,

$$\sigma^2 = 4N + 2 \sum_{i=1}^N \sum_{j=i+1}^N \text{cov}(-2 \ln p_i, -2 \ln p_j) \quad (4)$$

where p_i and p_j are the P -values for each test and the covariance (cov) is calculated as,

$$\text{cov}(-2 \ln p_i, -2 \ln p_j) = \rho_{ij} (3.25 + 0.75 \rho_{ij}), \quad \& \quad 0 \leq \rho_{ij} \leq 1 \quad \rho_{ij} (3.27 + 0.71 \rho_{ij}), \quad -0.5 \leq \rho_{ij} \leq 0 \quad (5)$$

with ρ_{ij} being the unadulterated correlation between variable i and variable j . Finally, the overall significance P of a set of non-independent tests is calculated using the statistic T which under the null hypothesis follows the central χ^2 distribution, where $T = T_0/c$ with $2N/c$ degrees of freedom and $c = \sigma^2/4N$.

Here, we test if each experimental measure aligns with a given model predicted state. Our null hypothesis is that the experimental measures do not align. P -values for individual variables, p_i , are calculated using two-sample t -tests between ill subjects and healthy controls. Where model predictions give a variable as high (+1), 'right-handed' one-tailed test are used, whereas a 'left-handed' test was used when model predictions are low (-1), to give the probability of obtaining the predicted value when the null hypothesis is true. For the case where the model predicts normal behavior for a variable (0) a two-tailed t -test is used. However, the p -value from the two-tailed test, $p_{\text{two-tail}}$, gives the probability that there is an observable difference between illness and control, which is the null hypothesis. To rectify

this, when comparing to a model predicted variable of 0 we take the P-value to be $p_i = 1 - p_{\text{two-tail}}$, giving the probability of obtaining the predicted value when the null hypothesis is true.

All cohort data was normalized using a Log2 transformation before T-tests and correlation calculations were performed. The unadulterated correlation values ρ_{ij} between two variables i and j were calculated in healthy subjects as the pairwise Pearson's linear correlation coefficient between variables. The above-mentioned experimental data was compared against model predictions based on the five measureable variables, namely TEST/EST, CORT, IIR, T1Cyt, and T2Cyt. Where model variables represent an aggregate set of markers each experimentally measured constituent marker was compared individually to the model predicted value. For example, T1Cyt is composed of IL-2, IFN γ and TNF β , therefore 3 individual P-values were calculated based on the predicted value of T1Cyt.

Results

Stable States in the HPA Models

Application of the discrete state representation to the basic stand-alone HPA model (Figure 1 A) generated 27 system states, and failed to produce multiple stable states (Figure 2). This is consistent with previous ordinary differential equation based models of this basic representation of the HPA axis [21-26]. Discrete state representation of the HPA-GR model (Figure 1 B) generated 243 system states. Of these, 2 system states possessed no outbound edges and were stable attractor steady states (Figure 2). In the first steady state all state variables assumed nominal values whereas the second steady state corresponded to activation of state variables GRD and GR with suppression of ACTH and CORT. Once again this solution is consistent with that obtained by analysis of the ordinary differential equation model of the HPA-GR system proposed by Gupta et al. [27] and Ben Zvi et al. [16].

Combining the HPA-GR axis with the HPG axis and immune system (Figure 1 B-G) altogether produced 4,782,969 system states. For the male HPG (model a) (Figure 1 C),

and three of the four female HPG models (models b, d and e) (Figure 1 D,E,G) five steady states were identified (Figure 2). One stable state is characterized by nominal values for all variables (SS0), which corresponds to the typically normal resting state of the system. The first alternate state (SS1) displays low ACTH with high GRD and GR, while the second (SS2) has inhibited innate and Th1 immune responses (low ICell, IIR, T1Cell, and T1Cyt), with increased Th2 activity (high T2Cell and T2Cyt). The third stable state (SS3) appears to be a combination of SS1 and SS2 with low ACTH, ICell, IIR, T1Cell and T1Cyt, and high GRD, GR, T2Cell and T2Cyt. The final state (SS4) presents with hypercortisolism, suppressed TEST and a shift towards the Th1 immune response (low T2Cell, T2Cyt, GnRH, LH/FSH and TEST/EST, and high CORT, GRD, GR, T1Cyt and T1Cell). The persistently low CORT state seen in the previous stand-alone HPA models of Gupta et al. [16] and Ben Zvi et al. [27], was not recovered here. Instead, CORT was expressed at a nominal or high value for all predicted states. SS1 most closely resembles the results of Gupta et al. [27], and Ben Zvi et al. [16], however these previous models only considered a single regulator of CORT, namely ACTH. The lack of a predicted hypocortisolic state in SS1 here can be attributed to the interplay of multiple regulators of CORT (ACTH, IIR, TEST/EST, and T1Cyt). Inclusion of additional regulators is not expected to further alter this state.

In the final female HPG model (model c) (Figure 1 F), corresponding to the ovulation phase, these same five states were recovered along with six new additional states (Figure 2). In the first three additional states the HPA axis and innate immune response are suppressed with low CRH, ACTH, CORT, ICell and IIR, while the HPG and anti-inflammatory response are raised with high T2Cell, T2Cyt, GnRH, LH/FSH and EST. The difference between the three states is noted in the level of glucocorticoid receptor response, GR and GRD, which together take values of low (SS5), nominal (SS6) and high (SS7). The remaining three additional states all give suppressed HPA (CRH, ACTH, and CORT) and lowered T1Cell activity, with high HPG activity (GnRH, LH/FSH and EST), and are again differentiated by their glucocorticoid receptor levels (GR, GRD): low (SS8), nominal (SS9) and high (SS10).

Overall, inclusion of the simplified immune system and the HPG works to regulate CORT levels in the HPA axis. The male HPG (HPG model a), and the majority of female HPG configurations (HPG models b, d and e), serve to produce either nominal values of CORT, with the potential of a shift towards Th2 activation (SS2 and SS3), or a hypercortisolic state with low TEST/EST and a shift towards Th1 (SS4). Only connections associated with the female gender (HPG model c) were responsible for the emergence of a natural hypocortisolic state (SS5 – SS10). This hypocortisolic state comes with high EST and may have a shift towards Th2 activation in the immune system.

Comparison of GWI and CFS to Predicted States

Application of Brown's meta-analysis method allowed for the calculation of a combined P-value comparing the experimental data with the predicted stable states, allowing for the alignment between different predicted stable states to be ranked. As experimental measures allowed for comparison with only five variables (TEST/EST, CORT, IIR, T1Cyt, and T2Cyt) several of the predicted stable states resulted in the same experimental profile and resulting combined P-value despite being distinct states (e.g. SS0 and SS1 both show nominal values for the five measureable variables).

To compare to our model the difference between steroid and cytokine levels recorded in male Gulf War veterans with GWI and HCs were compared to the steady state values predicted by the male variant of the HPA-GR-Immune-HPG model (model a). Comparison to the nominal states (SS0/SS1) showed poor alignment, $P_{SS0/SS1} = 0.82$, suggesting that the GWI profile cannot be considered the same as nominal behavior. Alignment with states presenting a shift towards Th2 immune activation (SS2/SS3) showed better alignment, $P_{SS2/SS3} = 0.38$, although with low significance. The final state, displaying hypercortisolism, low TEST and a shift towards Th1 immune activation (SS4), yielded the best alignment, $P_{SS4} = 0.30$, again however, with a low overall significance.

The difference between steroid and cytokine levels of female CFS subjects and HCs were compared to the steady state values predicted by the female variants of the HPA-GR-Immune-HPG models (model b-e). Again, alignment with states presenting nominal changes in measureable variables (SS0/SS1) was poor, $P_{SS0/SS1} = 0.83$, supporting that CFS is distinctly different from normal behavior. The Th2 shifted immune profile states (SS2/SS3) showed a significant alignment, $P_{SS2/SS3} = 0.04$, suggesting Th2 activation in CFS. This is further supported by low alignment with the Th1 immune activated state, with hypercortisolism, and low EST (SS4), $P_{SS4} = 0.28$. Improved alignment is seen in states with a shift towards Th2, coupled with hypocortisolism, and high EST (SS5/SS6/SS7), $P_{SS5/SS6/SS7} = 0.02$, suggesting that these features contribute to the CFS profile. This is also supported by low alignment with states only presenting hypocortisolism and high EST with no immune activation (SS8/SS9/SS10), $P_{SS8/SS9/SS10} = 0.60$.

Discussion

The existence of multiple stable states is a prime characteristic of systems incorporating feedforward and feedback mechanisms, and plays a critical part in guiding the complex dynamics observed in biology. These alternate stable regulatory regimes occur due to the feedforward and feedback mechanisms within the system and may allow escape routes for survival of an insult and provide support in the medium or long-term to what is equivalent to an uneasy cease-fire or adaptive compromise. An example of such compromises in functional status in exchange for survival include vasovagal response to decreased blood pressure and syncope ("fainting") [62]. From an evolutionary perspective it would be advantageous for a pathogen to establish an adaptive relationship with the host. As naturally occurring alternate states of homeostasis are inherently stable exploiting, these regimes could be an advantageous way for a pathogen to establish long-term chronic infection, in essence using the body's own homeostatic drive to maintain the status quo. To explore this hypothesis, we constructed a simple but integrated model incorporating three of the body's

major regulatory axes: the HPA, the HPG and the immune system. Modeling the dynamic properties of these complex systems presents a significant challenge, as much of the detailed information describing in vivo kinetics in humans is unavailable. However, there is a very significant body of connectivity data describing the interactions between the molecular and cellular elements of these biological systems. To make use of this wealth of information we have applied a discrete state representation to the neuroendocrine immune system based solely on the biological connectivity found in the literature and a set of ternary logical rules. Using a discrete logic methodology proposed by Thomas [30], we demonstrated that the inclusion of feedforward/feedback loops leads to multiple stable states. Indeed, addition of the positive feedback loop regulating glucocorticoid receptor dimerization (GR-GRD) to a basic model of the HPA axis generated an alternate homeostatic state characterized by high receptor expression and low circulating cortisol levels, a result found previously by Gupta et al. [27] and Ben Zvi et al. [16] using differential equation based models. So dependent is the natural emergence of these states on the regulatory wiring that inclusion of this receptor dimerization in a more complex HPA-Immune-HPG models resulted in the disappearance of this alternate hypocortisolic state through compensatory effects of these axes. Only when all three interacting axes were included was an alternate hypocortisolic condition recovered. Therefore while simple models require the inclusion of positive receptor feedback dynamics to produce multistability, these effects become inherent in more coarse, but comprehensive regulatory circuits, and receptor-level feedback becomes less of a contributor in the support of multiple attractor states. Coarse-grained but comprehensive models may suffice therefore in capturing physiologically relevant and clinically verifiable response dynamics.

Our analysis of these coarse grained models spanning across multiple regulatory axes highlighted the important role of gender in supporting a persistent hypocortisolic condition. Due to the suppressive actions of the male gonadal system in regulating itself and the HPA axis, a low cortisol steady state is never available to the male, at least theoretically at this level of detail. In women however, the combined effect of EST and PROG on the HPA still

remains somewhat inconsistent [34,63] owing to the varying effects of these hormones during and after the menstrual cycle. EST is generally believed to stimulate the HPA axis during the menstrual cycle [63-65], however evidence indicates that in perimenopausal, menopausal or ovariectomized women the HPA axis response is inversely correlated with plasma EST levels suggesting an inhibitory effect [65,66]. This suggests that sex hormone regulation may change in feedback polarity and act as both inhibitor and activator of the HPA axis. For this reason HPA-HPG interaction in women will in theory readily support the presence of a stable hypocortisolic condition when HPG axis regulation inhibits the HPA axis while stimulating itself.

In addition to sex hormone regulation, interaction with the immune system also appears to play a significant role in determining abnormal cortisol levels. In our coarse-grain models, cortisol exerts a suppressive action on the innate immune system and the Th1 adaptive immune response. Conversely, positive feedback by certain components of the immune system promotes increases in cortisol levels, which support a hypercortisolic steady state. While, inclusion of the glucocorticoid receptor dimerization (GR-GRD) in these models yielded additional steady states, it did not result in any significant changes to the profile in regards to cortisol levels. Combining the actions of HPA, HPG and immune regulation supported the existence of a stable hypercortisolic state in all models of men and women while a persistent hypocortisolic state was available only in women and only under certain modes of HPG regulation. Once again, while the inclusion of the GR-GRD receptor dimerization in this overarching model yielded additional steady states, it did not result in any significant changes to the homeostatic profiles.

These findings suggest that abnormally high levels of cortisol and adaptive immune activation, in this case Th1, may be perpetuated under certain conditions by the system's own homeostatic drive. This prediction of persistent and stable Th1 activation is consistent with evidence of anomalies in immune signaling in GWI [55,67,68]. Skowera et al. measured

intracellular production of cytokines in peripheral blood and found ongoing Th1-type immune activation in symptomatic Gulf War Veterans compared to healthy counterparts [67]. More recent work confirmed this finding while also suggesting that this may occur in the more complex context of a mixed Th1:Th2 response [55], something not captured by the simple immune model used here. Though we were unable to find documented reports of lower testosterone levels in GWI beyond the experimental data presented here, a large study of gulf war veterans in the UK found increased risk of fertility problems in this population [69], suggesting a possible relation.

In much the same way, conditions involving hypocortisolism and a Th2 shift may also be perpetuated at least in part by the natural homeostatic regulatory programming. In this case the homeostatic program may be driven by sex steroid suppression of the HPA axis and promotion of HPG function coupled with the mutual inhibition between the Th1 response and function of the gonadal axis, a configuration seemingly available only to female subjects in our models. This would suggest that the hypocortisolism seen in diseases, such as CFS [70-72], could be a result of the complexity afforded by the interaction between the HPA, immune and HPG axes in female subjects. Indeed model predictions describing such an alternate homeostatic state in women aligned with our experimental results from CFS subjects, and is consistent with previous findings of Th2 activation in CFS (Brenu et al., 2011, Nakamura et al., 2010 and Natelson et al., 2005, Broderick et al., 2010). This alignment with a naturally occurring homeostatic conditions may explain, at least in part, the biased prevalence of such persistent diseases in women [73-78]. Indeed, these authors report that approximately 70% of observed CFS patients are women. Additionally, the prevalence of CFS in the 40–49-year-old age range [78], and the higher prevalence of gynecological conditions and gynecological surgeries in women with CFS [79] supports the evidence that HPA suppression by estradiol appears more likely in perimenopausal, menopausal or ovariectomized women [65,66]. Interestingly, as many as 1 in 3 CFS subjects have reported symptom relief during pregnancy [80]. The normal trend in

pregnancy towards increased cortisol levels, especially in the third trimester, might be a contributing factor that would support the key involvement of sex hormone regulation proposed by our analysis [81]. While, in normal pregnancy this increase in cortisol typically coincides with an increase in cortisol-binding globulin (CBG) maintaining the level of free cortisol, CBG genetic variants in CFS have the potential to alter normal CBG function [82,83].

While certainly more comprehensive than their predecessors, these models remain relatively coarse representations of the interplay between the endocrine and immune systems. This is particularly true of immune model granularity, especially when one considers the complex signaling network supported by immune cells as well as other immune-sensitive cells [84]. The important role of key neurotransmitters linking the central nervous system with the HPA axis and the immune system was also under-represented in this first generation of models. For example, norepinephrine and epinephrine stimulate the β_2 -adrenoreceptor-cAMP-protein kinase A pathway inhibiting the production of Th1/proinflammatory cytokines and stimulating the production of Th2/anti-inflammatory cytokines causing a selective shift from cellular to humoral immunity [85,86]. Additionally, lymphocytes express most of the cholinergic components found in the nervous system. Lymphocytes may be stimulated by, or release, acetylcholine thus constituting an immune regulating cholinergic system secondary to the nervous system [87]. Another neurotransmitter, neuropeptide Y (NPY), also serves as a powerful immune modulator [88] and has recently been shown to play a role in CFS [89]. These components are without question important, however based on our initial observations from this piecewise analysis we expect that increased detail will lead to the emergence of additional response programs rather than the elimination of attractors found here.

As these models are based on currently documented knowledge of human physiology and regulatory biochemistry they are necessarily incomplete. Nonetheless the simple models

presented here illustrate the importance of an integrative approach to understanding complex illnesses. Further refinement of the model to include more detailed description of interactions within and between the HPA, HPG and immune systems could extend its applicability to other illnesses as would the incorporation of other key systems such as the brain and central nervous systems. Yet, even with the coarse-grained co-regulation networks investigated we found numerous stable resting states that differ significantly from normal and were indicative of complex and persistent regulatory imbalances. Findings such as this support the use of an alternate model for disease, one which is not necessarily associated with failure of individual components, but rather with a shift in their coordinated actions away from normal regulatory behavior. Response to exercise and other stressors has the potential to be very different in these new regulatory regimes. This is something that we have observed firsthand in our work with human GWI and CFS subjects [90].

Finally, when considering alignment with the experimental data presented here for CFS and GWI, it is important to remember that it was never our hypothesis that these illnesses resulted solely from the actions of homeostatic drive. Instead we proposed that homeostatic drive might be a significant contributor to the persistence of illness mechanisms. Because these naturally occurring regimes, once instantiated, provide an alternate stable homeostasis resistant to change, it may offer fertile ground in support of many chronic pathological processes. The alignment of several immune and endocrine markers modeled here with experimental data from CFS and GWI, two chronic conditions, would support at least partial involvement of the body's own homeostatic drive in facilitating the perpetuation of these conditions. This may promote resistance to therapy and the natural regulatory barrier to change, even positive change, should at least be considered in the design of robust treatment avenues.

Acknowledgments

This research was conducted in collaboration with Dr. Joel Zysman, Director of High Performance Computing, using the Pegasus platform at the University of Miami Center for Computational Science (CCS) (<http://ccs.miami.edu>). This research was also enabled by the use of computing resources provided by WestGrid and Compute/Calcul Canada.

References

1. Groeneweg FL, Karst H, de Kloet ER, Joëls M (2011) Rapid non-genomic effects of corticosteroids and their role in the central stress response. *J Endocrinol* 209: 153–167.
2. Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat Rev Neurosci* 10: 434-445.
3. Mikics É, Kruk MR, Haller J (2004) Genomic and non-genomic effects of glucocorticoids on aggressive behavior in male rats. *Psychoneuroendocrino* 29: 618–635.
4. Pariante CM, Lightman SL (2008) The HPA axis in major depression: classical theories and new developments. *Trend Neurosci* 31: 464 – 468.
5. Gerritsen L, Comijs HC, van der Graaf Y, Knoop AJ, Penninx BW, Geerlings MI (2011) Depression, hypothalamic pituitary adrenal axis, and hippocampal and entorhinal cortex volumes--the SMART Medea study. *Biol Psychiatry* 70: 373-380.
6. Mehta D, Binder EB (2012) Gene × environment vulnerability factors for PTSD: The HPA-axis. *Neuropharmacol* 62: 654-662.
7. Yehuda R (2009) HPA alterations in PTSD. In: Fink G, editor. *Stress Consequences: Mental, Neuropsychological and Socioeconomic*. San Diego: Academic Press. pp. 125-130.

- 629 8. Young EA (2009) PTSD and HPA axis: same hormones, different disorders. In: Pariante
630 CM, editor. *Understanding Depression: A Translational Approach*. New York: Oxford
631 University Press. pp. 193-212.
- 632 9. Gil-Bea FJ, Aisa B, Solomon A, Solas M, del Carmen Mugueta M, et al. (2010) HPA
633 axis dysregulation associated to apolipoprotein E4 genotype in Alzheimer's disease. *J*
634 *Alzheimers Dis.* 22: 829-838.
- 635 10. Golier JA, Schmeidler J, Legge J, Yehuda R (2006) Enhanced cortisol suppression to
636 dexamethasone associated with Gulf War deployment. *Psychoneuroendocrinology*
637 31(10): 1181-1189.
- 638 11. Golier JA, Schmeidler J, Legge J, Yehuda R (2007) Twenty-four hour plasma cortisol
639 and adrenocorticotrophic hormone in Gulf War Veterans: relationships to posttraumatic
640 stress disorder and health symptoms. *Biol Psychiatry* 62(10): 1175–1178.
- 641 12. Golier JA, Schmeidler J, Yehuda R (2009) Pituitary response to metyrapone in Gulf War
642 veterans: relationship to deployment, PTSD and unexplained health symptoms.
643 *Psychoneuroendocrinology* 34(9): 1338-1345.
- 644 13. Van Den Eede F, Moorkens G, Van Houdenhove B, Cosyns P, Claes SJ (2007)
645 Hypothalamic-pituitary-adrenal axis function in chronic fatigue syndrome.
646 *Neuropsychobiol* 55: 112–120.
- 647 14. Crofford LJ, Young EA, Engleberg NC, Korszun A, Brucksch CB, et al. (2004) Basal
648 circadian and pulsatile ACTH and cortisol secretion in patients with fibromyalgia and/or
649 chronic fatigue syndrome. *Brain Behav Immun* 18: 314–325.
- 650 15. Aschbacher K, Adam EK, Crofford LJ, Kemeny ME, Demitrack MA, et al. (2012) Linking
651 disease symptoms and subtypes with personalized systems-based phenotypes: A proof
652 of concept study. *Brain Behav Immun.* 26(7):1047-1056..

- 653 16. Ben-Zvi A, Vernon SD, Broderick G (2009) Model-Based Therapeutic Correction of
654 Hypothalamic-Pituitary-Adrenal Axis Dysfunction. *PLoS Comp Biol* 5: e1000273.
- 655 17. Alon U. Network motifs: theory and experimental approaches. *Nat Rev Genet.* 2007
656 Jun;8(6):450-61. Review.
- 657 18. Lightman SL, Conway-Campbell BL (2010) The crucial role of pulsatile activity of the
658 HPA axis for continuous dynamic equilibration, *Nat Rev Neurosci* 11: 710-718.
- 659 19. Bairagi N, Chatterjee S, Chattopadhyay J (2008) Variability in the secretion of
660 corticotropin-releasing hormone, adrenocorticotrophic hormone and cortisol and
661 understandability of the hypothalamic-pituitary-adrenal axis dynamics--a mathematical
662 study based on clinical evidence. *Math Med Biol* 25: 37–63.
- 663 20. Kyrylov V, Severyanova LA, Vieira A (2005) Modeling robust oscillatory behavior of the
664 hypothalamic-pituitary-adrenal axis. *IEEE Trans Biomed Eng* 52: 1977–1983.
- 665 21. Savic D, Jelic S (2005) A theoretical study of hypothalamo-pituitary adrenocortical axis
666 dynamics. *Ann NY Acad Sci* 1048: 430–432.
- 667 22. Savic D, Jelic S (2005) A mathematical model of the hypothalamopituitary-
668 adrenocortical system and its stability analysis. *Chaos Solitons Fractals* 26: 427–436.
- 669 23. Lenbury Y, Pornsawad P (2005) A delay-differential equation model of the feedback-
670 controlled hypothalamus-pituitary-adrenal axis in humans. *Math Med Biol* 22: 15–33.
- 671 24. Gonzalez-Heydrich J, Steingard RJ, Kohane I (1994) A computer simulation of the
672 hypothalamic-pituitary-adrenal axis. *Proc Annu Symp Comput Appl Med Care* 1994:
673 1010.
- 674 25. Dempsher DP, Gann DS, Phair RD (1984) A mechanistic model of ACTH-stimulated
675 cortisol secretion. *Am J Physiol* 246: R587-R596.

- 676 26. Sharma DC, Gabrilove JL (1975) A study of the adrenocortical disorders related to the
677 biosynthesis and regulation of steroid hormones and their computer simulation. Mt Sinai
678 J Med 42: S2-S39.
- 679 27. Gupta S, Aslakson E, Gurbaxani BM, Vernon SD (2007) Inclusion of the glucocorticoid
680 receptor in a hypothalamic pituitary adrenal axis model reveals bistability. Theor Biol
681 Med Model 4: 8.
- 682 28. Alon U (2007) Network motifs: theory and experimental approaches. Nat Rev Genet 8:
683 450-461.
- 684 29. Thomas R, Thieffry D, Kaufman M (1995) Dynamical behaviour of biological regulatory
685 networks--I. Biological role of feedback loops and practical use of the concept of the
686 loop-characteristic state. Bull Math Biol. 57: 247-276.
- 687 30. Thomas R (1991) Regulatory Networks Seen as Asynchronous Automata: A Logical
688 Description. J Theor Biol 153: 1-23.
- 689 31. Mendoza L, Xenarios I (2006) A method for the generation of standardized qualitative
690 dynamical systems of regulatory networks Theor Biol Med Model 3: 13.
- 691 32. Keller-Wood ME, Dallman MF (1984) Corticosteroid inhibition of ACTH secretion.
692 Endocrinol Rev 5: 1-24.
- 693 33. Viau V (2002) Functional Cross-Talk Between the Hypothalamic-Pituitary-Gonadal and -
694 Adrenal Axes. J Neuroendocrinol 14: 506-513.
- 695 34. Dallman MF, Viau V, Bhatnagar S, Laugero K, Gomez F, et al. (2002) Corticotropin-
696 releasing factor (CRF), corticosteroids and stress, energy balance, the brain and
697 behavior. In: Pfaff DW, editor. Hormones, Brain and Behavior. New York: Academic
698 Press. Pp. 571-632.

- 699 35. Tilbrook AJ, Turner AI, Clarke IJ (2000) Effects of stress on reproduction in non-rodent
700 mammals: the role of glucocorticoids and sex differences. *Rev Reprod* 5: 105–113.
- 701 36. Torpy DJ, Chrousos GR (1996) The three-way interactions between the hypothalamic-
702 pituitary-adrenal and gonadal axes and the immune system. *Baillieres Clin Rheumatol*
703 10: 181-198.
- 704 37. Rivier C, Rivest S (1991) Effect of stress on the activity of the hypothalamic pituitary-
705 gonadal axis: peripheral and central mechanisms. *Biol Reprod* 45: 523–532.
- 706 38. Piñón R (2001) *Biology of human reproduction*. Sausalito: University Science Books.
707 535 p.
- 708 39. Hiller-Sturmhöfel S, Bartke A (1998) The endocrine system: an overview. *Alcohol Health*
709 *Res World* 22:153-164.
- 710 40. Folcik VA, Broderick G, Mohan S, Block B, Ekbote C, Doolittle J et al. (2011) Using an
711 agent-based model to analyze the dynamic communication network of the immune
712 response. *Theor Biol Med Model* 8: 1.
- 713 41. Silverman MN, Pearce BD, Biron CA, Miller AH (2005) Immune modulation of the
714 hypothalamic-pituitary-adrenal (HPA) axis during viral infection. *Viral Immunol* 18: 41-
715 78.
- 716 42. Cutolo M (2004) Immune System, Hormonal Effects on. In: Martini L, editor.
717 *Encyclopedia of Endocrine Diseases*, Volume 2. Elsevier Academic Press. pp. 755-760.
- 718 43. Berczi I, Szentivanyi A (2003) Autoimmune disease. In: Berczi I, Szentivanyi A, editors.
719 *Neuroimmune Biology: Vol. 3: The Immune-Neuroendocrine Circuitry. History and*
720 *Progress*. Amsterdam: Elsevier Science BV. pp. 495-536

- 721 44. Bush KA, Krukowski K, Eddy JL, Janusek LW, Mathews HL (2012) Glucocorticoid
722 receptor mediated suppression of natural killer cell activity: Identification of associated
723 deacetylase and corepressor molecules. *Cell Immunol* 275:80-89.
- 724 45. Zen M, Canova M, Campana C, Bettio S, Nalotta L, et al. (2011) The kaleidoscope of
725 glucocorticoid effects on immune system. *Autoimmun Rev* 10: 305-310.
- 726 46. Liberman AC, Druker J, Refojo D, Holsboer F, Arzt E (2009) Glucocorticoids inhibit
727 GATA-3 phosphorylation and activity in T cells. *FASEB J* 23: 1558-1571.
- 728 47. Elenkov IJ (2004) Glucocorticoids and the Th1/Th2 balance. *Ann NY Acad Sci* 1024:
729 138-146.
- 730 48. González D, Díaz B, Pérez M, Hernández A, Chico B, et al. (2010) Sex hormones and
731 autoimmunity. *Immunol Lett* 133: 6-13.
- 732 49. Lélou K, Laffont S, Delpy L, Paulet PE, Périnat T et al. (2011) Estrogen Receptor α
733 Signaling in T Lymphocytes Is Required for Estradiol-Mediated Inhibition of Th1 and
734 Th17 Cell Differentiation and Protection against Experimental Autoimmune
735 Encephalomyelitis. *J Immunol* 187: 2386-2393.
- 736 50. Foster S, Daniels C, Bourdette D, Bebo BF (2003) Dysregulation of the hypothalamic
737 pituitary-gonadal axis in experimental autoimmune encephalomyelitis and multiple
738 sclerosis. *J Neuroimmunol* 140: 78-87.
- 739 51. Watanobe H, Hayakawa Y (2003) Hypothalamic interleukin-1 beta and tumor necrosis
740 factor-alpha, but not interleukin-6, mediate the endotoxin-induced suppression of the
741 reproductive axis in rats. *Endocrinology* 144: 4868-4875.
- 742 52. Fukuda K, Nisenbaum R, Stewart G, Thompson WW, Robin L, et al. (1998) Chronic
743 multisymptom illness affecting Air Force veterans of the Gulf War. *J Am Med Assoc* 280:
744 981-998.

- 745 53. Fukuda, K., Straus, S.E., Hickie, I., Sharpe, M.C., Dobbins, J.G., Komaroff, A., 1994.
746 The chronic fatigue syndrome: a comprehensive approach to its definition and study.
747 International Chronic Fatigue Syndrome Study Group. *Ann. Intern. Med.* 12, 953-959.
- 748 54. Collins JF, Donta ST, Engel CC, Baseman JB, Dever LL, et al. (2002) The antibiotic
749 treatment trial of Gulf War Veterans' Illnesses: issues, design, screening, and baseline
750 characteristics. *Control Clin Trials* 23: 333–353.
- 751 55. Broderick G, Kreitz A, Fuite J, Fletcher MA, Vernon SD, et al. (2011) A pilot study of
752 immune network remodeling under challenge in Gulf War Illness. *Brain Behav Immun*
753 25: 302–313.
- 754 56. Vernon SD, Reeves WC (2006) The challenge of integrating disparate high-content
755 data: epidemiological, clinical and laboratory data collected during an in-hospital study
756 of chronic fatigue syndrome. *Pharmacogenomics* 7: 345–354.
- 757 57. Fuite J, Vernon SD, Broderick G (2008) Neuroendocrine and immune network re-
758 modeling in chronic fatigue syndrome: An exploratory analysis. *Genomics* 92: 393–399.
- 759 58. Broderick G, Craddock RC, Whistler T, Taylor R, Klimas N, et al. (2006) Identifying
760 illness parameters in fatiguing syndromes using classical projection methods.
761 *Pharmacogenomics* 7: 407–419.
- 762 59. Broderick G, Fuite J, Kreitz A, Vernon SD, Klimas N, et al. (2010) A formal analysis of
763 cytokine networks in Chronic Fatigue Syndrome. *Brain Behav Immun* 24: 1209-1217.
- 764 60. Reeves WC, Lloyd A, Vernon SD, Klimas N, Jason LA, et al. (2003) Identification of
765 ambiguities in the 1994 chronic fatigue syndrome research case definition and
766 recommendations for resolution. *BMC Health Serv Res* 3: 25.
- 767 61. Brown M (1975) A method for combining non-independent, one-sided tests of
768 significance. *Biometrics* 31: 987–992.

- 769 62. Beacher FDCC, Gray MA, Mathias CJ, Crichton HD (2009) Vulnerability to simple faints
770 is predicted by regional differences in brain anatomy, *NeuroImage* 47: 937-945.
- 771 63. Wolfram M, Bellingrath S, Kudielka BM (2011) The cortisol awakening response (CAR)
772 across the female menstrual cycle. *Psychoneuroendocrinol* 36: 905-912.
- 773 64. Chrousos GP (2010) Stress and Sex Versus Immunity and Inflammation. *Sci Signal* 3:
774 pe36
- 775 65. Young EA, Korszun A, Figueiredo HF, Banks-Solomon M, Herman JP (2007) Sex
776 Differences in HPA axis regulation, In: Becker JB, Berkley KJ, Geary N, Hampson E,
777 Herman JP, et al., editors. *Sex Differences in the Brain: From Genes to Behavior*. New
778 York, USA: Oxford University Press. pp. 95-105.
- 779 66. Tarín JJ, Hamatani T, Cano A (2010) Acute stress may induce ovulation in women.
780 *Reprod Biol Endocrinol* 8: 53.
- 781 67. Skowera A, Hotopf M, Sawicka E, Varela-Calvino R, Unwin C et al. (2004) Cellular
782 Immune Activation in Gulf War Veterans. *J Clin Immunol* 24: 66-73.
- 783 68. Whistler T, Fletcher MA, Lonergan W, Zeng XR, Lin JM, et al. (2009) Impaired immune
784 function in Gulf War Illness. *BMC Medical Genomics* 2: 12.
- 785 69. Doyle P, Maconochie N, Ryan M (2006) Reproductive health of Gulf War veterans.
786 *Philos Trans R Soc Lond B Biol Sci* 361:571-584.
- 787 70. Papadopoulos AS, Cleare AJ (2012) Hypothalamic–pituitary–adrenal axis dysfunction in
788 chronic fatigue syndrome. *Nat Rev Endocrinol* 8: 22-32
- 789 71. Jerjes WK, Peters TJ, Taylor NF, Wood PJ, Wessely S, et al. (2006) Diurnal excretion of
790 urinary cortisol, cortisone, and cortisol metabolites in chronic fatigue syndrome.
791 *Psychosom Res* 60: 145–153.

- 792 72. Cleare AJ (2003) The neuroendocrinology of chronic fatigue syndrome. *Endocr Rev* 24:
793 236–252.
- 794 73. Nacul LC, Lacerda EM, Pheby D, Champion P, Molokhia M, et al. (2011) Prevalence of
795 myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) in three regions of
796 England: a repeated cross-sectional study in primary care. *BMC Med* 9: 91.
- 797 74. Evengard B, Jacks A, Pedersen N, Sullivan PF (2005) The epidemiology of chronic
798 fatigue in the Swedish Twin Registry. *Psych Med* 35: 1317-1326.
- 799 75. Gallagher AM, Thomas JM, Hamilton WT, White PD (2004) Incidence of fatigue
800 symptoms and diagnoses presenting in UK family care from 1990 to 2001. *J Roy Soc*
801 *Med* 97: 571-575.
- 802 76. Reyes M, Nisenbaum R, Hoaglin D, Unger ER, Emmons C, et al. (2003) Prevalence
803 and incidence of chronic fatigue syndrome in Wichita, Kansas. *Arch Intern Med* 163:
804 1530-1536.
- 805 77. Lindal E, Stefansson JG, Bergmann S (2002) The prevalence of chronic fatigue
806 syndrome in Iceland—a national comparison by gender drawing on four different
807 criteria. *Nord J Psychiatry* 56: 273-277. Erratum in: Lindal E, Stefansson JG, Bergmann
808 S (2006) *Nord J Psychiatry*. 60: 183.
- 809 78. Jason LA, Richman JA, Rademaker AW, Jordan KM, Plioplys AV, et al. (1999) A
810 community-based study of chronic fatigue syndrome. *Arch Intern Med* 159: 2129-2137.
- 811 79. Boneva RS, Maloney EM, Lin JM, Jones JF, Wieser F, et al. (2011) Gynecological
812 History in Chronic Fatigue Syndrome: A Population-Based Case-Control Study. *J*
813 *Womens Health* 20: 21-28.
- 814 80. Schacterle RS, Komaroff AL (2004) A comparison of pregnancies that occur before and
815 after the onset of chronic fatigue syndrome. *Arch Intern Med* 164:401-404.

- 816 81. Gangestad SW, Caldwell Hooper AE, Eaton MA (2012) On the function of placental
817 corticotropin-releasing hormone: a role in maternal-fetal conflicts over blood glucose
818 concentrations. *Biol Rev Camb Philos Soc* 87:856-873.
- 819 82. Torpy DJ, Ho JT (2007) Corticosteroid-binding globulin gene polymorphisms: clinical
820 implications and links to idiopathic chronic fatigue disorders. *Clin Endocrinol (Oxf)*
821 67:161-167.
- 822 83. Gagliardi L, Ho JT, Torpy DJ (2010) Corticosteroid-binding globulin: the clinical
823 significance of altered levels and heritable mutations. *Mol Cell Endocrinol* 316: 24-34.
- 824 84. Frankenstein Z, Alon U, Cohen IR (2006) The immune-body cytokine network defines a
825 social architecture of cell interactions. *Biol Direct* 1: 32.
- 826 85. Kin NW, Sanders VM (2006) It takes nerve to tell T and B cells what to do. *J Leukocy*
827 *Biol* 79: 1093-1104.
- 828 86. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES (2000) The Sympathetic Nerve—An
829 Integrative Interface between Two Supersystems: The Brain and the Immune System.
830 *Pharmacol Rev* 52: 595-638.
- 831 87. Kawashima K, Fujii T (2003) The lymphocytic cholinergic system and its contribution to
832 the regulation of immune activity. *Life Sci* 74: 675–696.
- 833 88. Wheway J, Mackay CR, Newton RA, Sainsbury A, Boey D, Herzog H, Mackay F (2005)
834 A fundamental bimodal role for neuropeptide Y1 receptor in the immune system. *J Exp*
835 *Med* 202:1527-1538.
- 836 89. Fletcher MA, Rosenthal M, Antoni M, Ironson G, Zeng XR, et al. (2010). Plasma
837 neuropeptide Y: a biomarker for symptom severity in chronic fatigue syndrome. *Behav*
838 *Brain Funct.* 6: 76.

90. Broderick G, Fletcher MA, Gallgher M, Barnes Z, Vernon SD, et al. (2012) Exploring the Predictive Potential of Immune Response to Exercise in Gulf War Illness. In: Yan Q. (Ed.), Psychoneuroimmunology: Methods and Protocols (Methods in Molecular Biology), Springer, New York, NY, In Press

Tables

Table 1: Ternary HIGH/LOW PASS operator

$A \nabla B$	$B = -1$	$B = 0$	$B = 1$
$A = -1$	0	0	-1
$A = 0$	0	0	-1
$A = 1$	1	1	0

Table 2: Ternary OR operator

$A \vee B$	$B = -1$	$B = 0$	$B = 1$
$A = -1$	-1	0	1
$A = 0$	0	0	1
$A = 1$	1	1	1

Table 3: Ternary NOT operator

A	$\neg A$
-1	1
0	0
1	-1

Figure Legends

Figure 1: Standard and extended HPA models. (A) Standard HPA model. (B) HPA-GR model of Gupta et al. [27]. Integrated models (C) HPA-GR-Immune-HPGa for males, and (D) HPA-GR-Immune-HPGb, (E) HPA-GR-Immune-HPGc, (F) HPA-GR-Immune-HPGd, and (G) HPA-GR-Immune-HPGe for females.

Figure 2: Steady states of standard and extended HPA models. White – nominal state (0); Green – high state (1); Red – low state (-1); Grey – N/A to the model.

Figure 1

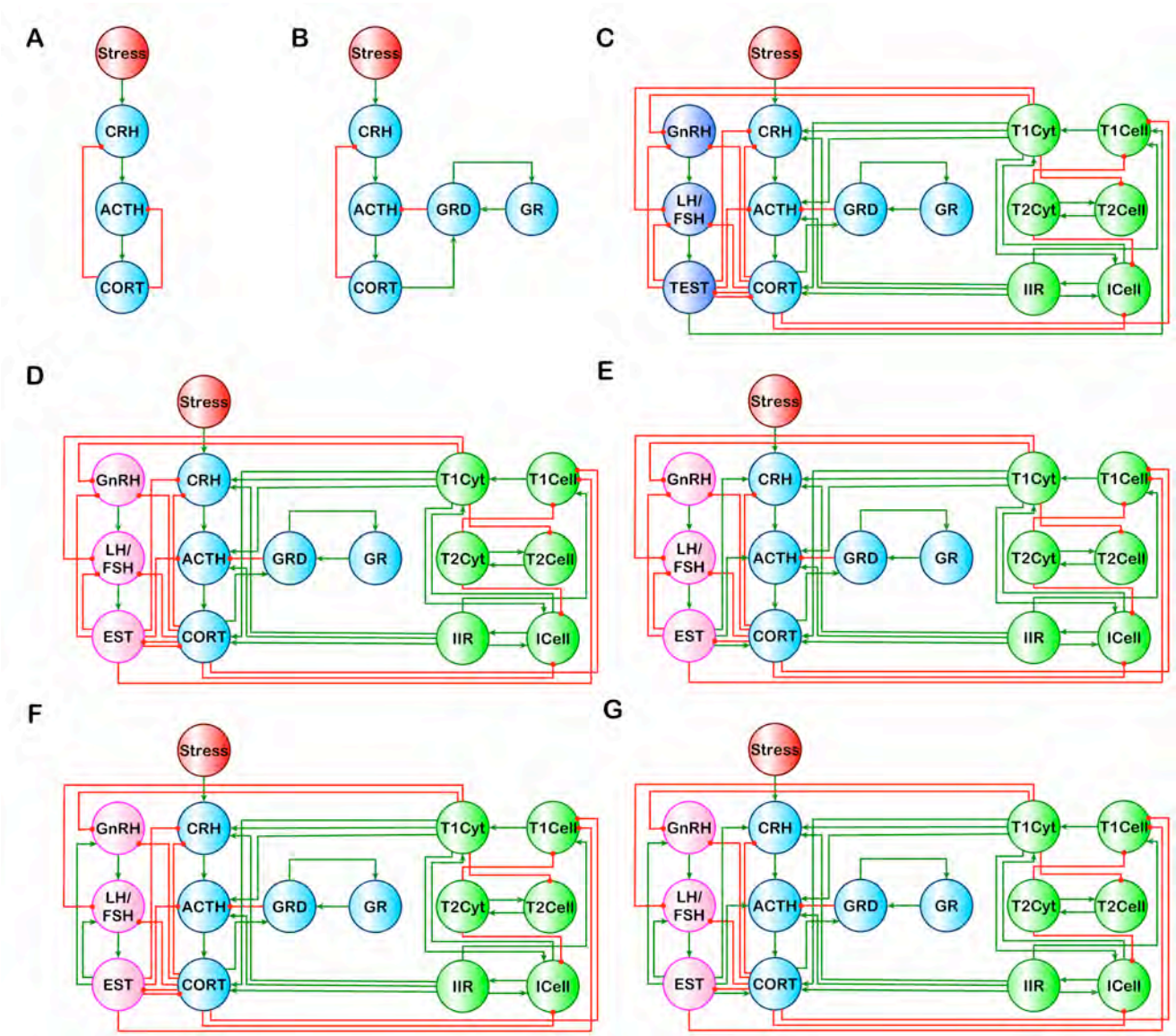


Figure 2



Appendix B:

Updated scope of work (SOW) submitted as part of request for transition of award to Nova Southeastern University.

Revised Statement of Work (SOW) - Relocation to Nova Southeastern University (NSU), FL

The following SOW has been updated to reflect current status of project consistent with the annual progress report submitted in September, 2012. Described are the remaining activities required for completion. These will now be conducted by the Broderick group from its new home institution: Nova Southeastern University, Fort Lauderdale, Florida.

In support of Dr. Broderick's transition Nova Southeastern University is entering into a service agreement with the Center for Computational Sciences (CCS) at the University of Miami, which will serve as the principal high-performance computing resource for the Broderick group from this point forward. The latter will continue to use the University of Alberta's WestGrid high-performance computing platform during the transition period in order to ensure continuity of the work.

Task 1. *Evaluate and select agent-based simulation environment.* **Completed.**

Task 2. *Define and encode immune cell populations and interaction rules.* **Completed.**

Task 3. *Refine HPA axis model and integrate with immune model.* **Completed.**

MILESTONE I: Completion and release of validated model combining ODE representation of the HPA axis and a discrete population-based model of the immune system. Target date: **Completed.**

Extensions to original Task 2, 3.

- 3.a. *Extension of circuit model of neuro-inflammatory cascades.* In an extension of the original mandate for Task 2, the circuit logic approach is also being applied to model inflammatory processes occurring in the brain and involving the cell types and immune signaling specific to this physiological compartment.
- 3.b. *Extension to sex hormone and thyroid axes.* Task 3 has also been extended beyond the original mandated scope to now include the endocrine axis regulating sex hormones. We expect to integrate thyroid function in this regulatory circuitry as well.

Timeline extended mandate: Months 1-4, Year 3 (now December, 2013)

Site(s): Nova Southeastern University

Task 4. *Design and conduct formal sensitivity and multi-stability analyses.* **Completed**

Task 5. *Network analysis of alternate homeostatic states.* **Completed**

Extensions to original Task 4, 5. These steps will be repeated in the analysis of the extensions to the model proposed in 3(a) and 3(b).

Timeline extended mandate: Months 1-4, Year 3 (now December, 2013)

Site(s): Nova Southeastern University

MILESTONE II: Verification of hypothesis that GWI symptoms persist because the endocrine-immune system now occupies and alternate homeostatic stable point and engages a new sub-optimal stress response control program.

Target date: Month 4, Year 3 (now December, 2013); Currently 70% complete.

Task 6. *Identify and deploy large-scale optimization.* This involves the selection of the best algorithm for exhaustive search of intervention possibilities. We expect the combined endocrine-immune system to present multiple stable points and the landscape describing its dynamic response to be complex. As a result standard techniques for optimization of treatment time course would terminate their search in the first region where treatment performance ceases to improve. Overall such a treatment may be quite remote from that available in the neighboring response "valley".

Timeline: Months 2-4, Year 3 (now October - December 2013)

- 6.a. *Review global search algorithms.* Review latest developments in evolutionary programming techniques as well as hybrid techniques to determine the most suitable search algorithm. Acquire or develop code and deploy on CCS platform and test on logic model developed in 4b.

Timeline: Month 1-2, Year 3 (now September - October 2013)

Site(s): Nova Southeastern University

- 6.b. *Configure simulation-based optimization scheme.* Configure an interface that evaluates the fitness of candidate interventions by repeatedly launching short logic model simulations as it conducts its search for the most robust treatment course. Test and deploy.

Timeline: Months 2-6, Year 3 (now October - February, 2014)

Site(s): Nova Southeastern University

Task 7. *Identify candidate treatment courses for GWI.* Using the optimization scheme developed and deployed in Task 6, launch optimization runs from multiple initial conditions of endocrine-immune status. Assess these options and report.

Timeline: Months 5-12 Year 3

- 7.a. *Define and encode solution fitness criteria.* Identify and describe mathematically the immune and endocrine descriptors that can be safely changed and over what range they may be changed. Incorporate these constraints with treatment goals and define optimization problem formally.

Timeline: Month 3-5, Year 3 (now November, 2013 - January, 2014)

Site(s): Nova Southeastern University

- 7.b. *Search for broadly applicable candidate treatment courses.* Identify a set of initial conditions of cytokine, hormone and immune cell abundance and launch repeated searches for optimal treatments from these points.

Timeline: Months 6-9, Year 3 (now February - May, 2014)

Site(s): Nova Southeastern University

- 7.c. *Critically assess candidate treatments.* Review candidate treatment courses and assess these critically based on efficacy and minimal invasiveness. Propose design of pilot clinical trials for evaluation of the best candidates.

Timeline: Months 10-11, Year 3 (now June - July, 2014)

Site(s): Nova Southeastern University

- 7.d. *End of project review and report.* Timeline: Month 12, year 3 (now August, 2014).